SAPIENZA
UNIVERSITÀ DI ROMA

Dottorato di Ricerca in

Tecnologie Biomediche Innovative in Medicina Clinica

- XXXIII ciclo -

Nuove frontiere nel carcinoma midollare della tiroide: identificazione di nuovi biomarkers diagnostici.

Novel frontiers in medullary thyroid cancer: identification of new diagnostic biomarkers.

Dottoranda: dr.ssa Valeria Ramundo
Matricola: 1446464

Tutor: prof. Cosimo Durante
Co-tutor: dr.ssa Antonella Verrienti

A.A. 2019/2020

Outline

Abstract

Background: medullary thyroid carcinoma (MTC) is rare thyroid cancer. The difficulty in management and the unfavorable outcome are mainly due to the absence of tools for early diagnosis. Recent studies have demonstrated the deregulation of microRNAs (miRNAs) in tumor samples from MTC patients. Extracellular plasma vesicles (pEVs) containing miRNA represent an emerging source of tumor biomarkers. There are no data from the circulating pEV-miRNAs in patients with MTC.

Aim: to identify new circulating diagnostic biomarkers in MTC.

Study design: multicenter, prospective, observational study enrolling adult patients with stage I-IV MTC (Group 1, n=23), compared to healthy subjects (Group 2, n=22). For Group 1 patients we collected: blood sample at the time of surgery and tumor tissue from the primary tumor. Step 1: pre-operative plasma from MTC patients (Group 1a) was compared to plasma from healthy subjects (Group 2). Step 2: pre-operative plasma from each MTC patients (Group 1a) was compared to tumor tissue of the same patient (Group 1b). Step 3: data from Step 1 and Step 2 were combined.

Methods: the expression analysis of circulating (pEV) and tissue miRNAs was performed by RT-qPCR with TLDA technology analyzing 754 miRNAs. Results were considered statistically significant when *P*-values were <0.05

Results: 555 miRNAs are considered to be informative comparing Group 1a and Group 2, 44 of them differentially expressed between two groups. 603 miRNAs are considered to be informative, comparing Group 1a *vs* Group 1b, 209 of them differentially expressed and 394 equally expressed between two groups, respectively. Combining Step 1 and Step 2 results, 24 miRNAs were identified.

Conclusions: a specific pattern of pEV-miRNAs was observed in MTC patients, which in part reflects tissue miRNAs arrangement. The 24 miRNAs identified represent potential diagnostic biomarkers in MTC, that could play an active role in the modulation of cellular pathways in targeted cells.

Medullary Thyroid Cancer

Epidemiology and diagnosis

Medullary thyroid carcinoma (MTC) was first described in 1959 as a distinct entity arising from the parafollicular C-cells of the thyroid, which synthesize and secrete calcitonin (Hazard, *et al.* 1959). The peculiar cellular origin of MTC renders it a special form of thyroid cancer with specific biological features and treatment needs that differ markedly from those of thyroid cancers with follicular-cell origins. MTC is a rare neuroendocrine tumor and accounts for only 3-5% of all thyroid malignancies, with an estimated incidence of 1400 new cases/year in the USA (Nelkin 2017; Wells, *et al.* 2015). Males and females are equally affected. The 10-year overall survival ranges from 40% (patients with distant metastases) to 75% (patients with locoregional disease) to 95% (patients with the intra-thyroid tumor) (Roman, *et al.* 2006).

The typical MTC presents as a single thyroid nodule, accompanied in 50% of cases by cervical lymphadenopathy, and sometimes associated with secretory diarrhea or other unusual hormonal effects. Diagnosis is generally based on the results of fine-needle aspiration biopsy (FNAB) and findings of elevated serum markers (calcitonin and carcinoembryonic antigen, CEA). Preoperative diagnosis is important because total thyroidectomy, followed by monitoring of serum calcitonin levels to detect recurrence, is the only curative option. Reoperation to correct the initial treatment increases morbidity and diminishes the chances of cure (Machens and Dralle 2013).

About 25% of MTC are hereditary autosomal dominant forms, which are associated with germline point mutations in the "REarrangement during Transfection" (*RET)* proto-oncogene and occur as part of the multiple endocrine neoplasia type 2 (MEN2), resulting in nearly complete penetrance of MTC in mutation carriers.

The MEN2 syndromes include two distinct subtypes, both characterized by strong genotype-phenotype correlations (Frank-Raue and Raue 2015). MEN2A, which accounts for 95% of MEN2 cases, and MEN2B, rarer and associated with particularly aggressive forms of MTC.

MEN2A includes four variants (Wells, *et al.* 2015). In the classic (and most common) form, MTC is frequently associated with primary hyperparathyroidism and pheochromocytomas. The incidence of the latter two conditions varies and depends on the *RET* codon that is mutated. In another variant, MEN2A occurs with cutaneous lichen amyloidosis, dermatological lesions that are particularly evident in the scapular region of the back, which corresponds to dermatomes T2–T6. The onset of these dermatological manifestations can occur early in life, before clinically evident MTC, and may thus serve as a precursor for the syndrome. In a further variant, MEN2A is associated with Hirschsprung's disease. Lastly, MTC can be the only clinical manifestation of the syndrome and this variant is known as familial MTC (FMTC).

MEN2B is characterized by a more uniform presentation: all patients develop MTC, ganglioneuromas of the aerodigestive tract, and a peculiar phenotype consisting of a marfanoid habitus, typical facies, and skeletal abnormalities. Over 60% of patients with MEN2B also develop pheochromocytomas (Wells 2018).

The remaining 75% of MTC are sporadic, with some *RET*-activating mutations identified in about 50% of cases, and somatic *HRAS* and *KRAS* mutations in 17-68% of *RET*-negative MTC patients (Ciampi, *et al.* 2012; Ciampi, *et al.* 2013; Moura, *et al.* 2011). *RET* mutations are associated with more aggressive disease in MTC, and thus tumors in a majority of patients with metastatic MTC harbor *RET* mutations (Ciampi, *et al.* 2019). Testing for the presence of germline *RET* mutations allows the identification of affected family members who will require prophylactic thyroidectomy. Most MTCs occur between the fourth and fifth decades of life (Elisei, *et al.* 2013a), although ages at diagnosis vary widely. MEN2-related MTCs occur early in life and are likely to be multifocal and bilateral. Sporadic forms are more common in older individuals and tumors are usually solitary and unilateral. Both sporadic and hereditary MTC is a typically round, circumscribed tumor located in the middle or upper third of the thyroid lobe, where the concentration of C-cell is highest. The tumor can present clinically with a morphological change of the thyroid, possibly palpable during physical examination but almost never causing any functional disorder of the thyroid. Advanced and metastatic

disease can be characterized by a functional syndrome, with diarrhea and other symptoms caused by the overproduction of calcitonin. Lymph node metastases are present at the time of diagnosis in 50-80% of patients with sporadic MTCs and in case-index patients with undiagnosed hereditary MTC. If cancer is suspected on the basis of clinical and/or ultrasound findings, an FNAB of the suspected thyroid nodule or regional lymph nodes should be submitted for cytology (Collins, *et al.* 1995). If sonographic (or clinical) findings are suspicious, contrast-enhanced computed tomography (CT) of the neck and chest is indicated (Wells, *et al.* 2015). Work-up for distant metastases is indicated if serum calcitonin levels exceed 500 pg/mL or clinical findings are suspicious. It should include chest CT, liver and axial bone Magnetic Resonance Imaging (MRI), and 6-fluoro-(18F)-L-dihydroxyphenylalanine (F-DOPA)-PET scan (if available) (Wells, *et al.* 2015).

Serum levels of calcitonin and CEA can provide valuable diagnostic, prognostic, and predictive information in patients with MTC. CEA is not a specific biomarker for the disease, but it is useful for detecting the progression of clinically evident MTC and for monitoring patients following surgery. As for serum calcitonin, preoperative levels are strongly correlated with tumor burden. Preoperative calcitonin levels are also useful for estimating the extent of lymph node metastasis and planning surgical management. Calcitonin and CEA doubling times are currently considered the best available indicators of MTC behavior, the likelihood of its recurrence, and the risk of cancer-related mortality (Laure Giraudet, *et al.* 2008; Meijer, *et al.* 2010). A calcitonin doubling time exceeding 6 months is associated with 5-year and 10-year survival rates of 92% and 37%, respectively. Shorter doubling times are associated with markedly lower survival rates (25% and 8% at 5 and 10 years, respectively) (Barbet, *et al.* 2005). In patients with poorly differentiated and aggressive MTCs, calcitonin values may actually decrease over time while CEA levels increase (Hadoux, *et al.* 2016).

The American Thyroid Association (ATA) lists the following points as objectives of the preoperative work-up of patients with possible MTC (Wells, *et al.* 2015):

1. Detection and quantitative assessment of any metastatic disease for surgical planning purposes.
2. In patients with MEN2, identification of concomitant conditions that could alter the surgical approach and surgical priorities, such as primary hyperparathyroidism and/or pheochromocytoma (Elisei, *et al.* 2013a).
3. Identification of germline *RET* mutations so that screening of the case index's first-degree relatives can be proposed for early diagnosis and treatment of affected individuals. Early genetic screening for patients harboring *RET* germline mutations results in an improvement in clinical presentation and morbidity (Ramundo, *et al.* 2011).

All MTCs should undergo AJCC/TNM staging, used for predicting disease-specific survival. The 8th edition, published in 2017, introduced several important changes in the staging criteria used to stage thyroid tumors, including MTCs. The most important involve the age cut-off for a low-risk status (from 45 to 55 years) and the reduced prognostic weight attached to microscopic extrathyroidal extension (Brierley, *et al.* 2017) (**Table 1** and **Table 2**). MTCs are solid, firm tumors that are generally not encapsulated. Calcifications are often present. Microscopic examination may reveal partial tumor encapsulation, but there is usually clear microscopic evidence of infiltration into the surrounding tissue. Amyloid deposition is typical but sometimes absent. Demonstration of calcitonin expression is mandatory for the diagnosis. Rare primary calcitonin-negative neuroendocrine carcinomas of the thyroid exist and must be distinguished from metastases from neuroendocrine lung tumors. In these cases, CEA determination can be useful, being the only neck tumor expressing this marker.

Table 1. AJCC 8th TNM staging system for MTC (from Amin, *et al.* 2017).

Primary tumor (pT)
Tx: tumor cannot be assessed
T0: no evidence of primary tumor
T1a: tumor ≤1 cm in greatest dimension limited to the thyroid **T1b**: tumor >1 cm but ≤2 cm in greatest dimension limited to the thyroid
T2: tumor >2 cm but ≤4 cm in greatest dimension limited to the thyroid
T3a*: tumor >4 cm limited to the thyroid **T3b*:** gross ETE invading only strap muscles (sternohyoid, sternothyroid, thyrohyoid, omohyoid) from a tumor of any size
T4: advanced disease **T4a**: moderately advanced disease; tumor of any size with gross ETE into the nearby tissues of the neck, including subcutaneous soft tissue, larynx, trachea, esophagus or recurrent laryngeal nerve **T4b**: very advanced disease; tumor of any size with extension toward the spine or into nearby large blood vessels, invading the prevertebral fascia or encasing the carotid artery or mediastinal vessels
Regional lymph node (pN)
Nx: regional lymph nodes cannot be assessed
N0a*: one or more cytologically or histologically confirmed benign lymph nodes **N0b*:** no radiologic or clinical evidence of locoregional lymph node metastasis
N1a*: ipsilateral or bilateral metastasis to level VI or VII (pretracheal, paratracheal, or prelaryngeal / Delphian, or upper mediastinal) lymph nodes; this can be unilateral or bilateral disease **N1b*:** metastasis to unilateral, bilateral, or contralateral lateral neck lymph nodes (levels I, II, III, IV, or V) or retropharyngeal lymph nodes
Distant metastasis (M)
M0: no distant metastasis
M1: distant metastasis

MTC, medullary thyroid cancer. ETE, extrathyroidal extension

*All categories may be subdivided in: (s) solitary tumor, (m) multifocal tumor. The largest tumor determines the classification

Table 2. AJCC prognostic staging for MTC (from Amin*, et al.* 2017).

Stage	**TNM**		
I	T1	N0	M0
II	T2	N0	M0
	T3	N0	M0
III	T1 – T3	N1a	M0
IVA	T4a	Any N	M0
	T1 – T3	N1b	M0
IVB	T4b	Any N	M0
IVC	any T	any N	M1

T, primary tumor. N, regional lymph node. M, distant metastasis.

Treatment

Total thyroidectomy offers the greatest chance for cure for MTC, and it remains the standard of care for the localized and oligometastatic disease, regardless of whether it is hereditary or sporadic. Depending on serum calcitonin levels and ultrasound findings, thyroidectomy should be accompanied by dissection of the cervical lymph node compartments. Given the complications associated with neck dissection procedures, removal of the necessary lymph node compartments should take place during the initial thyroidectomy, based on the frequency and pattern of lymph node metastasis relative to the location and size of the primary MTC, ultrasound findings, and serum levels of tumor calcitonin and CEA (Wells, *et al.* 2015). Like all patients who undergo total thyroidectomy, those operated on for MTC will need lifelong replacement therapy with levothyroxine. The dose should be adjusted to maintain serum thyrotropin levels within the normal range. There is no rationale for using TSH-suppressive therapy in thyroidectomized MTC patients since thyrotropin has no growth-stimulating effects on C-cells.

In patients with MEN2, total thyroidectomy is particularly important since MTC is typically multifocal and bilateral. MEN2 syndrome is characterized by a strong genotype-phenotype correlation (Raue and Frank-Raue 2018) and recommendations for the timing of surgery are based the classification of *RET* mutations into three risk levels (highest, high, moderate). Indeed, based on the *RET* germline mutation, according to the ATA guidelines, the risk of MTC aggressiveness can be defined as moderate, high, or highest according to the age at MTC onset and the presence of metastatic disease (Wells, *et al.* 2015). The ATA highest risk category includes patients with the *RET* M918T mutation. The ATA high risk category includes patients with *RET* codon C634 mutations and the *RET* codon A883F mutation. The ATA moderate risk category includes patients with *RET* codon mutations other than M918T, C634, and A883F (Wells, *et al.* 2015).

The onset of MTC is significantly age-related across the three *RET* mutational risk categories (Machens, *et al.* 2018). The most important prognostic factor in patients with MEN2 is tumor stage at diagnosis. After the initial signs of MTC, all hereditary MTCs progress with

comparable aggressiveness, regardless of the initial risk stratification (Raue and Frank-Raue 2018).

In patients with MEN2B, MTC often presents in infancy and metastasizes early to regional lymph nodes and distant sites. In most cases, however, the syndrome is sporadic, and the affected patient has a de novo *RET* mutation. Only 25% of cases occur in families with previous or current manifestations of MEN2B (Wells, *et al.* 2015). In MEN2B associated with the A883F mutation, the MTC appears to exhibit more indolent behavior than that associated with a *RET* M918T mutation (Jasim, *et al.* 2011).

The occurrence of multiple *RET* mutations (including the C634Y and M918T mutations) has been described in several MEN2 patients (Dvorakova, *et al.* 2006; Valente, *et al.* 2013). The effects of the different mutations can be additive. For example, the occurrence on the same allele of two mutations with relatively mild effects (V804M together with Y806C, S904C, E805K, or Q781R) is associated with a higher transforming capacity, peculiar of a MEN2B phenotype, than that seen with the single mutations (Wells, *et al.* 2015).

Early identification and risk classification of asymptomatic *RET* mutation carriers are crucial since prophylactic thyroidectomy is essential (Raue and Frank-Raue 2018). A "window of opportunity" has been described in which a total thyroidectomy alone can provide adequate therapy. Exploiting this window can eliminate the need for lymph node dissections, which increase surgical morbidity (e.g., transient or permanent hypoparathyroidism, laryngeal nerve palsy). If the window is missed and neck lymph node dissection is necessary, the chances of a biochemical cure are reduced (Machens and Dralle 2018). Regardless of whether the patient has MEN2A or MEN2B, the presence of pheochromocytoma should be excluded before any diagnostic or therapeutic intervention (Wells 2018).

For carriers of a *RET* codon 918 mutation (found in more than 95% of patients with MEN2B), surgery is recommended as soon as possible, ideally within the first year of life, despite the high operative risk. Achieving a surgical cure is more difficult after 4 years of age (Elisei, *et al.* 2012; Raue and Frank-Raue 2018). Dissection of the central lymph node compartment (level VI) depends on the ability to identify and preserve or transplant the parathyroid glands to reduce the risk of hypoparathyroidism (Wells, *et al.* 2015).

Children carrying the *RET* mutation 634 (located in the high-risk group) should undergo prophylactic thyroidectomy by the age of 5 years if not earlier. Timing decisions should be based on the results of serum calcitonin assays performed every 6 months. For subjects in the moderate-risk group, the timing of total thyroidectomy should be based on the detection of elevated (> 10 pg/ml) serum calcitonin levels. Measurement of serum calcitonin levels should be started around 5 years of age and repeated every 6-12 months (Wells, *et al.* 2015).

In patients with advanced or metastatic MTC, none of the systemic therapies currently approved have been shown to improve overall survival. Therefore, there is no evidence-based guidance on when to start these drugs and how patients with indolent disease should be followed. Multidisciplinary input for these decisions is strongly recommended to ensure optimal care for these patients. Active treatment (e.g., locoregional or systemic multikinase inhibitors administration) should be considered in the presence of symptoms, lesions close to vital structures, high-tumor burdens, or disease progression defined by Response Evaluation Criteria in Solid Tumors **(**RECIST v1.1**)** (Eisenhauer, *et al.* 2009).

Systemically administered chemotherapy (doxorubicin, alone or combined with cisplatin or 5-fluorouracil or dacarbazine) has historically yielded poor results in MTC (Hadoux and Schlumberger 2017). Vandetanib and cabozantinib are two multi-kinase inhibitors (MKIs) approved as first-line systemic treatments for patients with advanced and metastatic MTC, not eligible for surgical or other local treatment (Elisei, *et al.* 2013b; Wells, *et al.* 2012). However, their anti-tumor effects stem mainly from their strong inhibition of key angiogenic pathway components, including vascular endothelial growth factor receptor type 2 (VEGFR-2).

The phase III ZETA trial compared vandetanib (300 mg daily) with placebo in 331 patients with symptomatic and/or metastatic MTC, measurable disease by RECIST 1.1, and calcitonin levels of at least 500 pg/mL (Wells, *et al.* 2012). The predicted progression-free survival in the vandetanib arm significantly exceeded that of the placebo group (30.5 *vs* 19.3 months, $P<0.001$). The overall response rate was also significantly higher in the vandetanib arm compared with placebo (45% *vs* 13%, $P<0.001$). Although it was not designed to evaluate

overall survival, there was no difference in overall survival between the treatment groups. Radiological responses were accompanied by significant reductions in calcitonin and CEA levels in 69% and 52% of cases, respectively. Vandetanib activity was not dependent on the tumor's *RET* status, previous treatment, metastasis sites, disease progression status, or tumor burden. To date, there is no published evidence that vandetanib improves survival over that seen with placebo. Vandetanib has undergone phase I/II study in children with MTC. Partial responses were observed in 47% of the patients, and the adverse effects recorded resembled those reported in adult patients (Fox, *et al.* 2013).

The phase III EXAM trial compared cabozantinib (140 mg daily) with placebo (2:1) in 330 MTC patients with progressive disease prior to study enrollment (Elisei, *et al.* 2013b). Cabozantinib was associated with a longer median progression-free survival compared with placebo (11.2 *vs* 4.0 months, $P<0.001$) and a significantly higher rate of partial responses compared with placebo (28% *vs* 0%). The drug's efficacy was unrelated to patient age, tumor location or burden, progression rate, prior kinase inhibitor treatment (in 20% of patients) or *RET*/*RAS* mutation status. Median overall survival rates were similar in the cabozantinib and placebo arms (26.6 *vs* 21.1 months, $P=0.24$), but cabozantinib displayed significant superiority in the subgroup of patients with *RET* M918T mutation (Schlumberger, *et al.* 2017).

The ZETA and EXAM trials both focused on patients with advanced and/or metastatic MTC, but differences in the trials' designs and inclusion criteria preclude direct comparison of their findings. Clear evidence supporting vandetanib over cabozantinib as the first-line treatment in this setting is thus lacking. Possible factors to consider in choosing one or the other as first-line treatment might be based on potential toxicity in each patient. The survival advantages achieved with cabozantinib in patients with *RET* M918T or *RAS* mutations might also deserve some consideration (Schlumberger, *et al.* 2017).

MKIs are frequently associated with numerous side effects, including palmar-plantar erythrodysesthesia, photosensitivity, stomatitis, taste changes, hypertension, diarrhea, nausea, anorexia, fatigue, and uncontrolled hypothyroidism. In the ZETA trial, over 30% of patients receiving vandetanib experienced diarrhea, rash, nausea, and/or hypertension.

Corrected QT interval prolongation was a severe, unexpected side effect in 8% of cases (Bastholt, *et al.* 2016). Grade 3 or 4 adverse events also occurred in 69% of the EXAM subjects who received cabozantinib. The most common were diarrhea (15.9%), hand-foot syndrome (12.6%), and fatigue (9.3%). Cabozantinib is a more potent inhibitor of VEGFR-2, which leads to a higher risk for hemorrhage, venous thrombosis, intestinal perforation, and fistula formation. Thus, it is advised to implement a patient-focused evaluation when recommending a therapeutic agent that conveys oncologic benefit balanced by a lower burden of side effects (Cabanillas, *et al.* 2014).

Other anti-angiogenic MKIs (e.g., sorafenib, motesanib, pazopanib, sunitinib, lenvatinib) have already undergone phase II trials in advanced MTC patients. The most encouraging results concerned sunitinib and lenvatinib, which produced response rates of 50% and 36%, respectively (Hadoux and Schlumberger 2017). Thus far, however, no MKIs have been approved for second-line use in MTC.

There are two selective RET inhibitors currently under investigation in clinical trials for the management of tumors harboring RET mutations: pralsetinib (BLU-667) and selpercatinib (LOXO-292). These drugs are highly potent RET-selective protein tyrosine kinase inhibitors with anti-tumor effects in MTC, whereas their anti-angiogenic activity is negligible. Pralsetinib inhibits the protein product of RET M918T, as well as RETV804L/M gatekeeper mutations conferring resistance to tyrosine kinase inhibitors, while selpercatinib is a highly selective RET kinase inhibitor with nanomolar potency against the canonical RET MTC drivers, RET gatekeeper mutations, and RET fusions (Al-Jundi, *et al.* 2020). Preliminary data from the ongoing clinical trials documented minimal side effects, excellent tolerability, and high efficacy (Al-Jundi, *et al.* 2020; Subbiah, *et al.* 2018a; Subbiah, *et al.* 2018b; Wirth, *et al.* 2020).

Radionuclide therapy is another systemic treatment in selected cases of metastatic MTC patients. Yttrium-90-DOTA-[D-Phe1-Tyr3]-octreotide (^{90}Y-DOTATOC) was tested in a phase II trial that included 31 patients with metastatic MTC and increasing calcitonin levels. Post-treatment reductions in calcitonin levels occurred in 29% of the patients treated, and

survival benefits were also observed in the responders (Iten, *et al.* 2007). The efficacy of radionuclide therapy has not been compared with that of MKI. Ideally, this comparison should be done within a clinical trial setting. In short, there is little evidence supporting the use of chemotherapy or radionuclide therapy in patients with MTC, but either can be considered if MKIs are contraindicated.

Prognosis and follow-up

The correlation between MTC phenotypes and somatic *RET* mutations are less straightforward than those for their germline counterparts. Recent studies found that MTCs harboring a somatic *RET* mutation tended to be more advanced at the time of diagnosis more likely to recur, and associated with poorer survival, compared with *RAS*-mutated MTCs. The same results were obtained when considering only tumors with the *RET* M918T mutation, which is the most frequent somatic mutation (Vuong, *et al.* 2018).

Patients treated for MTC require lifelong follow-up. Serum calcitonin and CEA levels should be monitored during the early and long-term postoperative staging workups (Meijer, *et al.* 2010). Shorter follow-up might be justified if tumor progression is suspected. Serial measurements of tumor markers allow the calculation of doubling times, which provide useful information. Currently available data indicate that calcitonin doubling times should be based on at least 4 consecutive measurements, preferably obtained over a 2-year period, by the same laboratory using the same assay (Elisei, *et al.* 2013a; Wells, *et al.* 2015). Clinically relevant foci of the disease are rarely found in patients with calcitonin levels <150 pg/mL, but the likelihood of structural disease increases as calcitonin and CEA levels rise.

Multiple imaging modalities should be used to identify locoregional and/or distant metastases (Elisei, *et al.* 2013a; Wells, *et al.* 2015). Contrast-enhanced whole-body (brain, neck, thorax, abdomen, and pelvis) CT scans with ultra-thin reconstructions are sensitive and specific enough to allow one to estimate the burden of systemic disease and to assess and identify target lesions. Target and non-target lesions should be assessed using RECIST v1.1 (Eisenhauer, *et al.* 2009). Contrast-enhanced MRI is more sensitive in identifying liver

and brain lesions. Ultrasound is useful for assessing cervical lymph node involvement. Bone scans in MTC patients are high in sensitivity but low in specificity. Whole-body bone MRI without contrast medium offers higher specificity, particularly for osteoblastic lesions. Contrast enhancement is recommended when extraosseous extension or compression of the spinal cord or other neurological structures is suspected. Endoscopic exploration of the upper digestive tract and airways is mandatory when infiltration is suspected.

MTCs are generally relatively indolent tumors and consequently display low 18F-fluorodeoxyglucose (FDG) avidity. Therefore, 18F-FDG Positron emission tomography (PET)-CT is not recommended for their staging, but it can be more informative in cases of an advanced disease characterized by dedifferentiation and rapid progression (Hadoux, *et al.* 2016). F-DOPA-PET has displayed high sensitivity and specificity in MTC (Romero-Lluch, *et al.* 2017; Treglia, *et al.* 2012), but its high cost and limited availability currently preclude its use in clinical settings. Gallium-68 (^{68}Ga) somatostatin analog PET-CT is relatively insensitive and is not useful for staging. However, results reflect the expression of somatostatin receptors, which is useful information when the feasibility of radionuclide therapy is being explored (Bodei, *et al.* 2004).

Molecular biology

Dysregulation of multiple signaling pathways has been reported to contribute to the pathogenesis of MTC and is generally associated with the genetic alterations of genes involved in these pathways (Al-Jundi, *et al.* 2020).

To date, the molecular mechanisms of MTC tumorigenesis remain unclear.

RET gene

Over 95% of the hereditary MTCs and 35-50% of sporadic MTCs harbor gain-of-function mutations involving the *RET* proto-oncogene. Each class of mutations is associated with specific disease phenotypes and occurrence patterns (Mulligan 2014). The human RET protein has three isoforms that are distinguished from one another by their carboxy-

terminal amino acids. Upon activation, RET undergoes phosphorylation on multiple intracellular tyrosine residues which lead to the activation of numerous downstream signaling pathways that promote cell growth, proliferation, survival, or differentiation (Mulligan 2014).

Activation of wild-type *RET* requires the binding of a ligand and co-receptor complex. Different activation mechanisms have been identified in the mutant forms of RET found in MTCs. RET mutations in patients with FMTC can be located in either the extracellular cysteine-rich domain or the intracellular tyrosine kinase domain (Romei, *et al.* 2016a).

Extracellular domain mutations, which are found in MEN2A and FMTC, lead to the constitutive dimerization of mutant RET, with more intense activation of downstream pathways. The most frequent MEN2A-associated mutations affect the extracellular cysteine-rich domain codified by RET exons 10 and 11 and involve cysteine residues (e.g., cysteines at codons 634, 630, 611, 618, and 620) (Accardo, *et al.* 2017; Yeganeh, *et al.* 2015).

The FMTC-associated RET mutations that affect the intracellular domain seem to be characterized by ligand-independent activation. These proteins are phosphorylated and signal as monomers, and the process does not seem to be enhanced by the presence of ligands.

The RET mutations with the most severe effects (e.g., M918T), which is found in MEN2B, alter the conformation of the kinase, increasing its ATP-binding capacity and facilitating its constitutive activation. The kinase's activity can be further enhanced by the presence of the RET ligands, which suggests that it can be generated by monomeric forms or by dimers.

The majority (95%) of patients with MEN2B harbor a single germline mutation in exon 16 (M918T) (Accardo, *et al.* 2017). Roughly 5% have the A883F mutation, which affects exon 15 (Yeganeh, *et al.* 2015). In over 90–95 % of cases, MEN2B syndrome is caused by a de novo germline *RET* mutation, mainly in exon 15 or 16 (Frank-Raue and Raue 2015).

RET M918T also accounts for 75-95% of all somatic *RET* mutations (Chernock and Hagemann 2015).

Other genes and mechanisms involved in MTC

Somatic mutations involving the *RAS* genes are the second most common mutations in sporadic MTCs. Molecular data from several studies confirm that *RET* and *RAS* mutations are mutually exclusive. The prevalence of *RAS* mutations in *RET* wild-type MTCs ranges from 0% to 81.3% (Moura, *et al.* 2015). *HRAS* and *KRAS* are more frequently mutated than *NRAS* (Boichard, *et al.* 2012; Ciampi, *et al.* 2013; Moura, *et al.* 2015; Moura, *et al.* 2011; Romei, *et al.* 2016a).

Although the majority of MTC studies have not found *BRAF* mutations, commonly implicated in papillary thyroid cancer, a Greek and a Korean study have reported the BRAF V600E mutation in patients with MTC (Cho, *et al.* 2014; Goutas, *et al.* 2008; Ji, *et al.* 2015), and a BRAF fusion has been reported in a sporadic *RET* wild-type and *RAS* wild type MTC (Kasaian, *et al.* 2016).

Recent studies using next-generation sequencing technology has revealed the presence of several "non-canonical" gene alterations in MTCs. *CCND1, CCND2, CDK4, CDK6, CDKN2A/B,* or *CDKN2C*, involved in cell cycle regulation, were reportedly altered in 21% of primary MTCs or metastases (Heilmann, *et al.* 2016), although in all cases, the non-canonical mutations were found together with a known *RET* or *RAS* driver mutation.

Among the other molecular mechanisms involved in MTC tumorigenesis, there is the influence of several genetic modifiers such as *RET* polymorphisms, or other genetic or epigenetic alterations.

Several efforts have been made to identify the molecular event responsible for the 2-5% of MEN2 syndromes in which *RET* germline mutations are absent (Frank-Raue and Raue 2015; Romei, *et al.* 2016a).

MicroRNAs (miRNAs), characterized by short length (18-25 nucleotides), are a large subgroup of non-coding RNAs involved in the regulation of post-transcription pathways. Increasing evidence has shown that miRNAs participated in multiple biological processes, which were associated with the initiation and progression of tumors, such as tumor cell proliferation, metastasis, invasion, and apoptosis (Jiang, *et al.* 2019).

Recent studies have demonstrated the deregulation of miRNAs in tumor samples from MTC patients, with potential prognostic and therapeutic implications (Chu and Lloyd 2016).

MiRNA-183, miRNA-375, and miRNA-9* are able to discriminate between sporadic and hereditary MTC. MiRNA-183 and miRNA-375 are up-regulated whereas miRNA-9* is down-regulated in sporadic MTC compared to hereditary MTC (Abraham, *et al.* 2011). MiRNA-375, miRNA-21, miRNA-224, and miRNA-183 have also been shown to have an important prognostic role (Abraham, *et al.* 2011; Aubert, *et al.* 2018; Galuppini, *et al.* 2017; Mian, *et al.* 2012; Romeo, *et al.* 2018). One of the possible miRNA-183 targets is YAP1, a transcriptional coactivator that can exert both an oncogenic and tumor suppressive role (Hudson, *et al.* 2013). This is probably in accordance with the observation that MTC does not express YAP1 protein (Celano, *et al.* 2018; Galuppini, *et al.* 2017). MiRNA-127 seems to be correlated with *RET* status, as it is more overexpressed in *RET* wild-type sporadic MTC compared to *RET*-mutated sporadic MTC (Mian, *et al.* 2012).

MiRNA-182 expression is up-regulated in MTCs harboring *RET* mutations in codons 918 and 634 and it can promote a more aggressive phenotype, while higher miRNA-224 expression levels are correlated with the presence of *RAS* mutations and with a less aggressive phenotype (Cavedon, *et al.* 2017; Spitschak, *et al.* 2017).

The expression of three miRNA-processing genes (*EXPO5, DICER1,* and *DGCR8*) is significantly increased in MTCs harboring *RET* mutations, in particular RET634 (Puppin, *et al.* 2014).

Fu et al. investigated the miRNA profile in human MTC tissue and identified 32 differentially expressed miRNAs between MTC and healthy tissues. In particular, hsa-miR-1, hsa-miR-9-5p, and hsa-miR-195-3p might have the potential to be used as diagnostic and therapeutic targets of MTC (Fu, *et al*. 2017).

Unmet needs

The lack of tools for the early diagnosis, for the prediction of disease progression, and the prediction of the response to currently available treatments makes management of MTC

difficult and outcome unfavorable. Despite all these novelties, much work remains to be done to fully understand the pathogenesis and biological behaviors of MTC and to transfer this knowledge in clinical practice.

Hence the idea of a large multicenter research project focused on a rare disease, deriving from the need to identify new markers involved in the diagnosis, prognosis, and follow-up of MTC.

Liquid biopsy

Liquid biopsy is a non-invasive diagnostic tool for body fluid genotyping, very promising for diagnosis, prognosis, prediction, and monitoring of treatment response (Burrell and Swanton 2014; Prete, *et al.* 2020).

The liquid biopsy approach has been mainly concentrated on the analysis of circulating tumor cells, circulating tumor nucleic acids and/or tumor-derived extracellular vesicles, and tumor extrachromosomal circular DNA which have been shed from tumors, and their metastatic sites into the body fluids of cancer patients. Liquid biopsies can bring new insights into the intra-tumor heterogeneity and genetic and epigenetic alterations responsible for metastases and treatment efficacy. These genetic and epigenetic changes can be different in each individual and the liquid biopsy concepts can improve the personalized medicine approach (Khatami, *et al.* 2019).

Extracellular vesicles (EVs) are nano-biological units released from most cell types into the extracellular environment; they include exosomes and ectosomes. Afterward, they are taken up by neighboring or distant recipient cells. EV cargo includes lipids, proteins, and nucleic acids (DNA and several types of RNA). Several physiological functions of EVs are ascribed to cell-to-cell communication, such as favoring cellular differentiation and epithelial-mesenchymal transition, promoting angiogenesis, and modulating immune responses. EVs play also an important role in pathological conditions such as cancer. Thus, the EV-mediated crosstalk between cancerous and non-cancerous cells can modulate the biochemistry and consequently the function of stromal components to stimulate the growth, expansion, and

spread of cancer cells. Several reports support the concept that EVs also contribute to distant intercellular communication in cancer. Recently, Hoshino et al. showed that EV-associated integrins determine organ-specific metastasis through a selective adhesion of EVs to extracellular matrix-enriched cellular areas, followed by their uptake by resident cells at their predicted metastatic destination. The potential of EVs and their content as cancer biomarkers is increasingly being recognized. Tumor-derived EVs may be investigated for their protein expression or genetic profile as diagnostic or prognostic markers. A potential drawback of plasma/serum EV analysis is that they contain not only cancer-derived EVs, but also EVs released by blood cells, endothelial cells, stromal cells, and others. Moreover, during neoplastic growth, immune response and the often associated inflammation may alter the rate of release of EVs (Rappa*, et al.* 2019).

To date, there is no published data on plasmatic EV (pEV)-miRNAs in MTC.

Aims

This research project aimed to identify new circulating diagnostic biomarkers in MTC.

Primary objectives:

a) To identify the circulating pEV-miRNAs and tissue miRNAs in patients with MTC;

b) To identify MTC-specific pEV-miRNAs.

Materials and methods

Study subjects and study design

This is a multicenter, prospective, observational study enrolling patients with MTC from four Italian referral centers for MTC (Sapienza University, Roma; University of Siena; University of Pisa; Istituto Nazionale Tumori IRCCS, Pascale Foundation, Napoli).

A total of 23 adult (> 18 years old) patients with stage I-IV sporadic MTC, ECOG performance status ≤ 3 (Dilts, *et al.* 2008), were consecutively enrolled between April 2018 and September 2019 and underwent total thyroidectomy associated in most cases with neck dissection according to current guidelines. Subjects with a history of other malignant tumors were excluded.

A cohort of healthy control adult (> 18 years old) subjects was also enrolled at Sapienza University (Roma) to match MTC patients for gender and age distribution.

This study was approved by the Institutional Ethical Committee and written informed consent was provided by all recruited subjects.

All subjects were classified into two groups:

1. Group 1: 23 patients with MTC;
2. Group 2: 22 healthy subjects.

For patients of Group 1, we collected:

- A blood sample (plasma) at the time of surgery (pre-operative) (Group 1a);
- A tumor tissue sample from the primary tumor (7 Fresh-frozen, FF; 16 Formalin-fixed paraffin-embedded, FFPE) (Group 1b);

For subjects of Group 2, we collected a blood sample (plasma).

The study was conducted in the following steps:

1) Step 1: Pre-operative plasma from MTC patients (Group 1a) was compared to plasma from healthy subjects (Group 2), with the aim to identify pEV-miRNAs able to discriminate between the two conditions (MTC *vs* healthy subject).

2) Step 2: Pre-operative plasma from each MTC patient (Group 1a) was compared to tumor tissue of the same patient (Group 1b), with the aim to identify tumor-derived pEV-miRNAs.
3) Step 3: data from Step 1 and Step 2 were analyzed to identify pEV-miRNA as potential diagnostic biomarkers.

Plasma collection

Blood samples from MTC patients (Group 1a) and healthy controls (Group 2) were collected in an EDTA tube and processed within 1 hour from the collection. Samples were centrifuged at 3000 rpm for 10 minutes and plasma fraction was transferred to RNase-free tubes and stored at −80°C until RNA extraction. To eliminate the risk of bias related to hemolysis, all plasma samples were visually assessed and those that were hemolyzed, icteric, or lipemic were excluded from the analysis.

pEV isolation

Plasma samples were centrifugated at 10000 rpm for 10 minutes at 4°C, and the supernatant was transferred in new eppendorf without disturbing pellet to proceed with Exosomes Isolation Exosomes were isolated using Exoquick (System Bioscience Cat# EXOQ5A-1) and following the manufacturer's instructions. For defibrination of plasma samples, 8 µl of Thrombin (Ci=611 U/ml) (System Biosciences Cat# TMEXO-1) were added to 1mL of each plasma. Briefly, we mixed 500 µl of plasma with 4 µl of thrombin and incubated the samples at room temperature (RT) for 5 minutes. Samples were centrifuged for 5 minutes at 10000 rpm and the serum-like solutions supernatant was treated with 126 µl of precipitation buffer, flicked, and incubated at 4°C for 30 minutes. Samples were centrifuged twice at 1500 rpm, firstly for 30 minutes then for 5 minutes, before completely removing the supernatant. Finally, pellets were resuspended in 200 µl of phosphate-buffered saline (PBS).

pEV quality controls: Western Blot analysis

The isolated exosomes were lysed in the RIPA Buffer in the 1:1 ratio and subjected to western blot analysis (8% SDS-polyacrylamide gel electrophoresis; 40 μg protein/lane) using anti-TSG101 (Cat#HPA006161, Atlas Antibodies); anti-Calreticulin (Cat#PA3-900, ThermoFisher); anti-CD63 (Cat#VPA00798, Bio-Rad); anti-GAPDH (Cat#ab8245, Abcam). The proteins were visualized on the BioRad ChemiDoc MP Imaging System (BioRad, Hercules, CA).

RNA extraction

EV-RNA was extracted by using the Norgen plasma/serum RNA purification kit. Three synthetic spike-in (ath-miR-159a, cel-miR-254, osa-miR-414) for monitoring extraction efficiency were added to samples after lysis. Extracted RNA was eluted in 30 μl of H_2O RNase-free and subjected to quality control by RealTime PCR using the ratio of miR-23a to miR-451. A ratio of more than 7 is an indicator of possible hemolysis. 2 μl of RNA of each sample were processed for real-time PCR with TaqMan Advanced miRNA Array Cards (TLDA), following the manufacturer instructions.

Tissue sample

RNA extraction from FFPE and FF

Primary tumor archival FFPE tissue blocks were used for RNA extraction. From the FFPE blocks, two slides were cut by microtome: one hematoxylin and eosin (H&E) slide was reviewed by a specialist pathologist to identify and mark the tumor area and one 10-μm non-stained slides were used for RNA extraction. Non-stained slides were dewaxed using established protocols. The marked H&E slide was used as a guide for manual macrodissection of tissue from the non-stained slides. RNA was extracted using NORGEN Tissue Samples FFPE RNA Extraction Kit (Cat#25300), according to the manufacturer's instruction.

Trizol Reagent (Invitrogen, ThermoFisher Scientific, CA, USA) was used to isolate total RNA from fresh-frozen primary MTC tissue samples. To increase the RNA yield of the tissue samples, we added 250 μg glycogen (Invitrogen, ThermoFisher Scientific, CA, USA) for each milliliter of Trizol.

For both methods, during the extraction 5 μl of 400 pM solution of spike-in (ath-miR159a) were added to each sample. The extracted RNA was eluted in 30ul of H_2O RNase-free and quantity and quality were evaluated with a Nanodrop ND-100 spectrophotometer (ThermoFisher Scientific). Furthermore, samples were cleaned up from any chemical residues and genomic DNA using RNase-Free DNase treatment (Invitrogen, ThermoFisher Scientific, CA, USA) and QIAGEN miRNeasy micro-kit (Cat#74004).

For each sample, we reverse-transcribed 10 ng of total RNA using a TaqMan® Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, ThermoFisher Scientific).

MiRNA expression analysis

MiRNA expression profiling was performed on cDNA from the plasma (Group 1a, Group 2) and tissue samples (Group 1b) using RT-qPCR with TaqMan Advanced miRNA Human A and B cards (Applied Biosystems, ThermoFisher Scientific), which detect the 754 best characterized members of the human miRNA genome.

Data analysis was performed using the R environment (http://www.r-project.org/) for quality control, cleaning, normalization, and differential expression analysis. MiRNA expression levels were normalized by using "norm.rankinvariant" method, and relative levels were calculated with the comparative threshold cycle (Ct) method. MiRNAs with Ct values ≥33 were excluded.

MiRNA target and functional enrichment analysis

To provide biological insight of deregulated miRNAs, a functional enrichment analysis was performed: Gene Ontology (GO) of the biological process was assessed by using the cluster

Profiler R and the MIENTURNET (MicroRNA ENrichment TURned NETwork) tool (http://userver.bio.uniroma1.it/apps/mienturnet/) and miRNAs targets enrichment analysis was performed using the SpidermiR packages and the String tool (https://string-db.org/).

Statistical analysis

Statistical analysis was performed with RStudio (http://www.r-project.org/).

Differential expression analysis between groups was assessed with the Wilcoxon test and considered statistically significant when *P*-values were <0.05. A false discovery rate (FDR) adjusted *P*-value was calculated.

Heatmaps were generated in the R environment using differentially expressed miRNAs as input data.

Results

1. *Study population*

A total of 23 MTC patients (Group 1) and 22 healthy subjects (Group 2) were enrolled in the study. **Table 3** summarizes the characteristics of the study population at the time of the enrollment.

Table 3. Characteristics of study population at the time of the enrollment.

	Group 1 (n=23)	**Group 2 (n=22)**
Sex		
Females – n (%)	12 (52.2)	12 (54.5)
Males – n (%)	11 (47.8)	10 (45.5)
Age in years—median [range]	51 [23-74]	48.5 [21-75]
Stage		
I – n (%)	11 (47.8)	-
II - n (%)	1 (4.3)	
III - n (%)	3 (13.1)	
IVA – n (%)	0	
IVB – n (%)	0	
IVC – n (%)	2 (8.7)	
NA – n (%)	6 (26.1)	

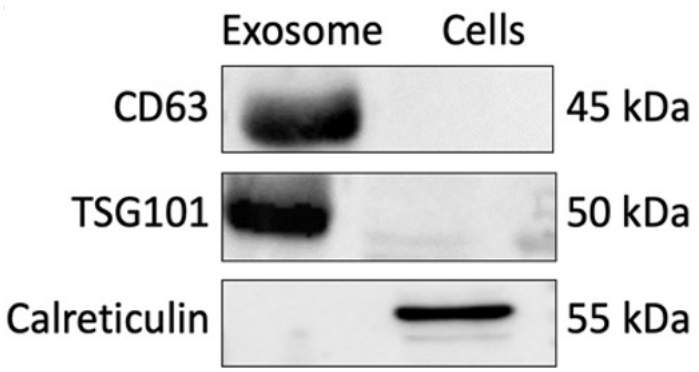
MTCs were classified and staged according to the 8th edition of TNM staging (Brierley, *et al.* 2017).

NA, not applicable (preoperative staging not performed).

2. *Exosome isolation assay*

Exosomes extracted from plasma (pEV) express two specific markers (CD63 and TSG101) and do not express Calreticulin, indicating that the exosome preparation is devoid of cellular contaminants (**Figure 1**).

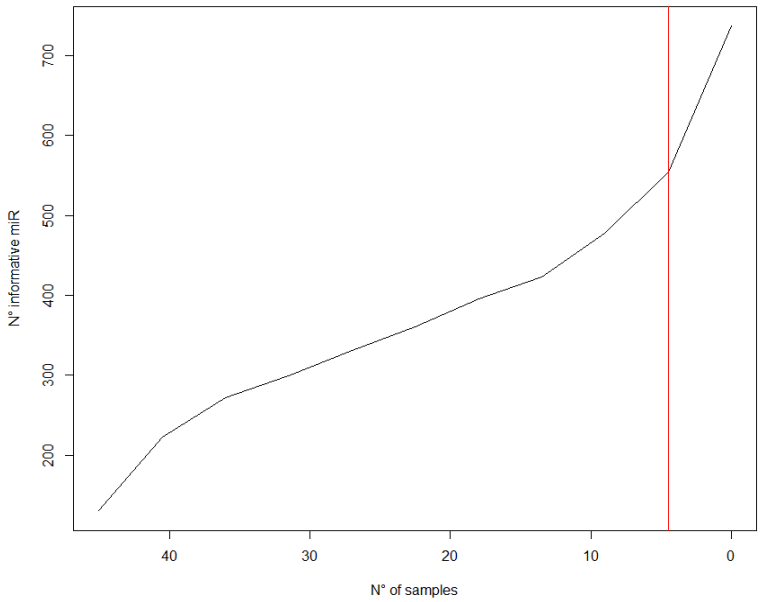
Figure 1. Western Blot for exosomes extracted from plasma.



3. *Step 1: identification of differentially expressed pEV-miRNAs in MTC patients (Group 1a) vs healthy subjects (Group 2)*

Starting from the analysis of the 754 miRNAs, only miRNAs with Ct <33 present in at least 10% of the samples were considered informative. A total of 555 miRNAs were identified to be informative comparing Group 1a and Group 2 (**Figure 2**).

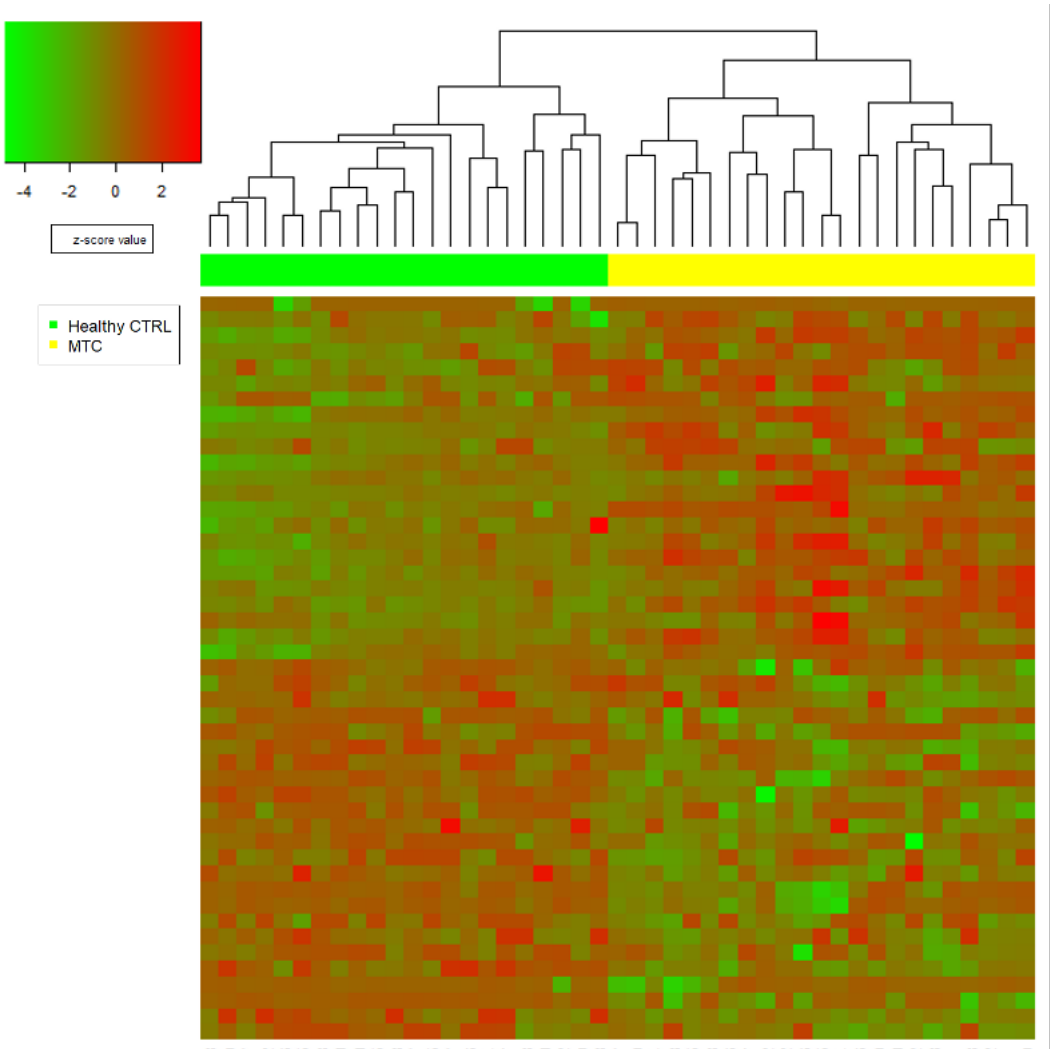
Figure 2. Informative miRNAs between MTC patients (Group 1a) and healthy subjects (Group 2).



	nInformativeMiR	perc
0%	737	0.0
10%	555	4.5
20%	478	9.0
30%	423	13.5
40%	396	18.0
50%	360	22.5
60%	331	27.0
70%	299	31.5
80%	272	36.0
90%	222	40.5
100%	130	45.0

Differential expression analysis of the levels of the 555 miRNAs expressed in the plasma of MTC patients (Group 1 a) and healthy subjects (Group 2) allowed the identification of 44 miRNAs deregulated in the two groups. Hierarchical clustering analysis of these differentially expressed miRNAs and plasma samples are presented in **Figure 3**.

Figure 3. Heatmap of the differentially expressed 44 pEV-miRNAs in Group 1 *vs* Group 2. The upper color bar represents sample classes; yellow represents MTC patients blood samples and green represents healthy controls blood samples.



Among these 44 differentially expressed pEV-miRNAs, 21 are up-regulated and 23 are down-regulated (**Table 4**).

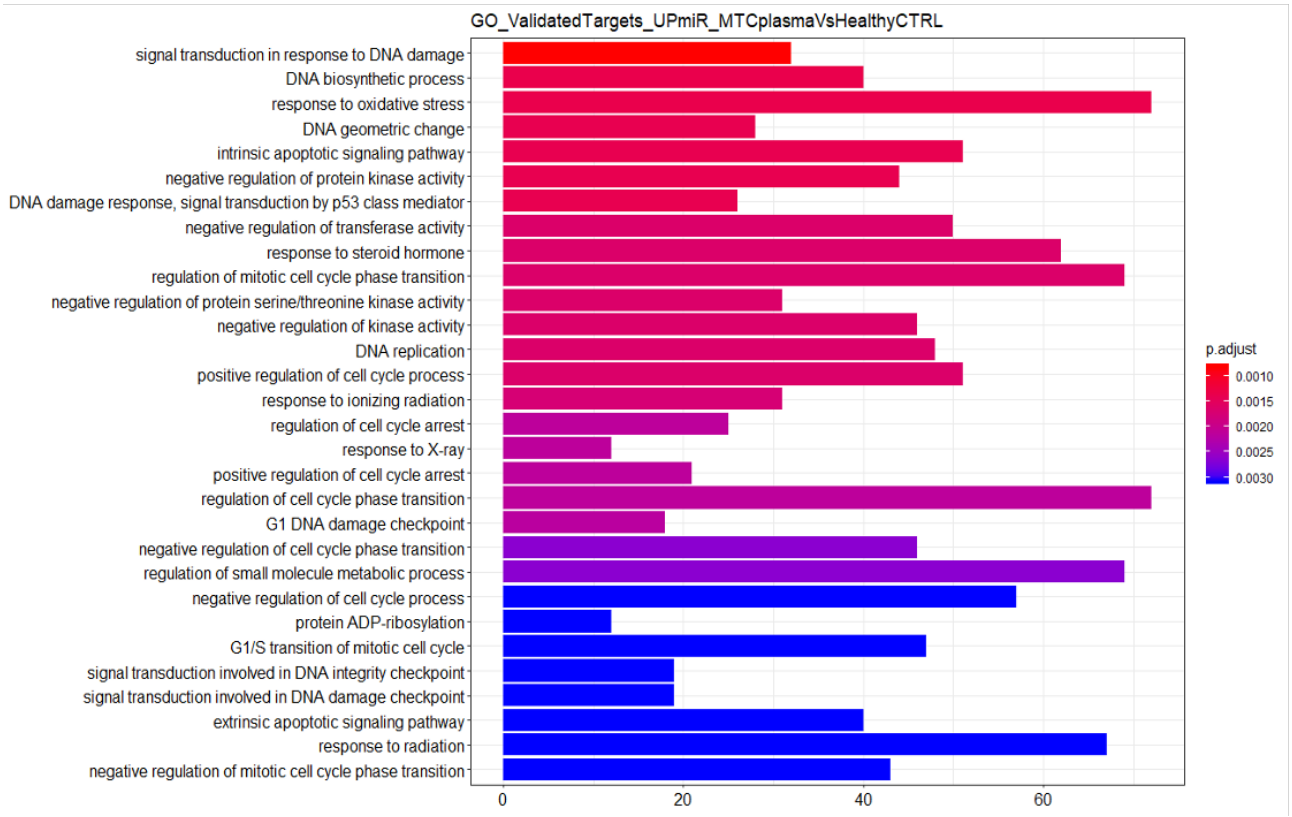
Table 4. Up-regulated and down-regulated pEV-miRNAs in Group 1a *vs* Group 2.

Up-regulated miRNAs	**FC**	***P*-value**		**Down-regulated miRNAs**	**FC**	***P*-value**
hsa_miR_7_2_3p_478199_mir	2.186425	3.685759e-02		hsa_let_7d_3p_477848_mir	0.4275519	1.013915e-09
hsa_miR_18b_5p_478584_mir	2.4725862	0.0000475085		hsa_let_7f_1_3p_477801_mir	0.4177361	5.011423e-03
hsa_miR_26b_5p_478418_mir	2.352378	2.069781e-07		hsa_miR_10b_5p_478494_mir	0.4402351	8.435165e-05
hsa_miR_101_5p_478620_mir	4.068403	7.146216e-06		hsa_miR_24_3p_477992_mir	0.3702297	7.880448e-07
hsa_miR_187_3p_477941_mir	3.285145	0.006275258		hsa_miR_25_3p_477994_mir	0.4443446	0.0001310693
hsa_miR_197_3p_477959_mir	2.088678	0.03685759		hsa_miR_30a_5p_479448_mir	0.1948327	0.0166012
hsa_miR_223_3p_477983_mir	2.046947	0.007811237		hsa_miR_100_5p_478224_mir	0.4679071	5.7199e-08
hsa_miR_329_3p_478029_mir	2.194029	0.02428157		hsa_miR_125b_5p_477885_mir	0.4542059	2.105999e-08
hsa_miR_370_3p_478326_mir	2.842276	0.01554938		hsa_miR_183_5p_477937_mir	0.3370588	0.006275258
hsa_miR_373_3p_478363_mir	2.233253	0.0009511994		hsa_miR_193a_5p_477954_mir	0.3963082	5.7199e-08
hsa_miR_412_3p_478087_mir	8.459697	0.001040995		hsa_miR_215_5p_478516_mir	0.4659102	1.986602e-06
hsa_miR_432_5p_478101_mir	2.410153	0.009009578		hsa_miR_217_478773_mir	0.3958361	0.02428157
hsa_miR_450b_3p_478913_mir	2.1379466	0.0309259076		hsa_miR_222_3p_477982_mir	0.4919951	1.067426e-05
hsa_miR_454_3p_478329_mir	2.527203	0.03092591		hsa_miR_296_5p_477836_mir	0.4203733	2.668218e-06
hsa_miR_513a_5p_479483_mir	3.034215	0.01110731		hsa_miR_320a_478594_mir	0.4504792	3.869493e-08
hsa_miR_520d_5p_478616_mir	2.222132	0.0002005003		hsa_miR_362_3p_478058_mir	0.4688562	0.00367805
hsa_miR_548b_5p_478589_mir	2.477608	0.0007922786		hsa_miR_423_5p_478090_mir	0.4754735	1.986602e-06
hsa_miR_548j_5p_479022_mir	4.448282	0.04370825		hsa_miR_450b_5p_478914_mir	0.3217898	0.0004945102
hsa_miR_574_3p_478163_mir	2.578618	3.323003e-05		hsa_miR_532_5p_478151_mir	0.3640722	5.689487e-09
hsa_miR_654_3p_479135_mir	5.881785	0.0003689354		hsa_miR_617_479100_mir	0.0166012	0.3607044
hsa_miR_770_5p_479178_mir	7.336757	7.536026e-05		hsa_miR_660_5p_478192_mir	0.3143299	2.799319e-09
				hsa_miR_1208_478637_mir	0.3261872	0.02144138
				hsa_miR_1275_477890_mir	0.05044284	0.03012268

FC, fold-change.

The enrichment analysis of the biological processes for the experimentally validated targets of the up-regulated miRNAs revealed that these miRNAs could have several effects on target cells. **Figure 4** shows the biological processes in order of significance. In particular, among those enriched in a more significant way, there are the cellular response to DNA damage, the process of DNA biosynthesis, the response to oxidative stress, the intrinsic pathway of apoptosis, the negative regulation of protein kinase activity, and the cell cycle regulation.

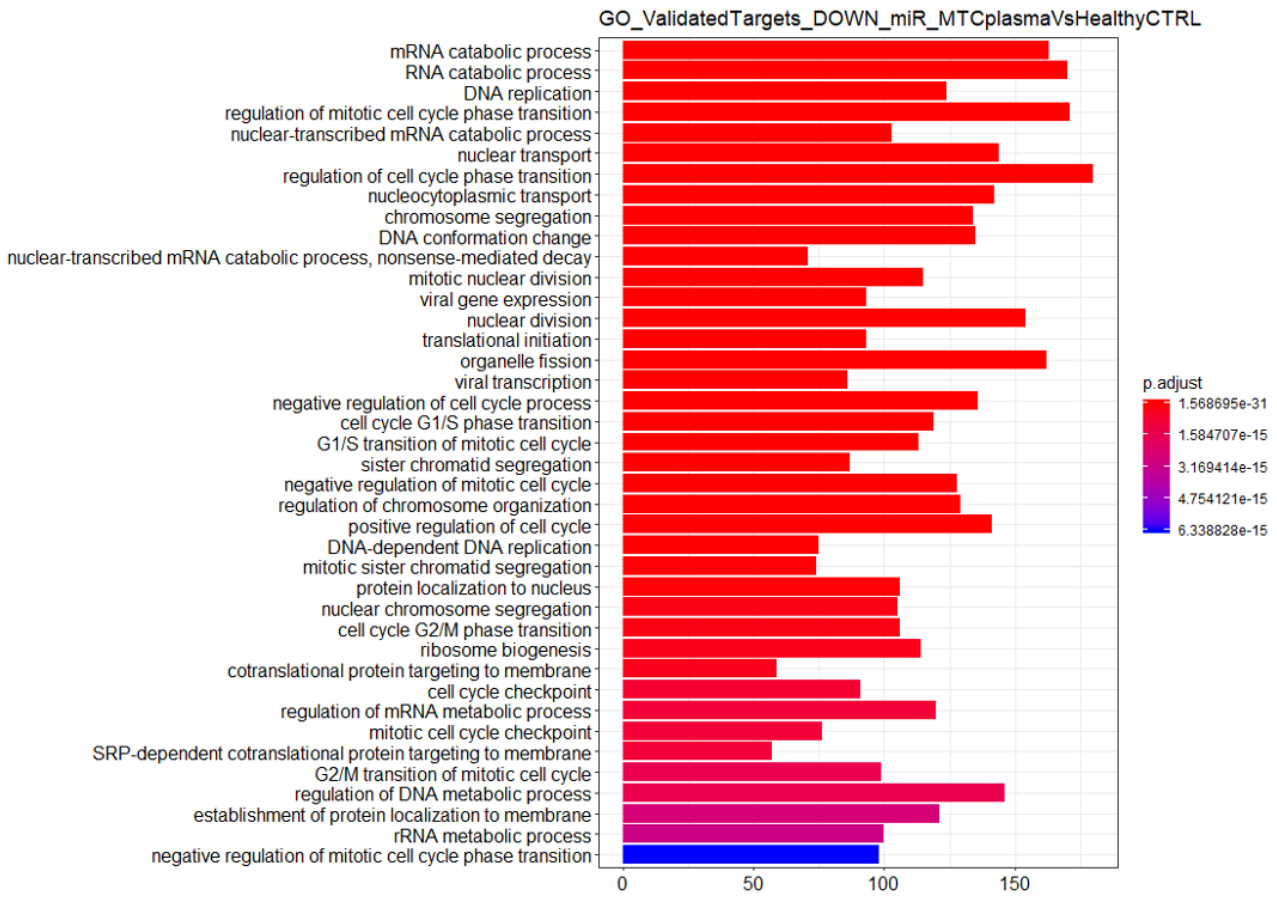
Figure 4. Enrichment analysis of the biological processes for the experimentally validated targets of the up-regulated miRNAs.



GO, gene ontology.

We repeated the same enrichment analysis for the down-regulated miRNAs targets. **Figure 5** shows the biological processes in order of significance. In particular, the biological processes most likely to be altered by miRNAs seem to be RNA catabolism, DNA replication, cell cycle regulation, and nuclear transport. It is interesting to note the highly statistically significant FDR values.

Figure 5. Enrichment analysis of the biological processes for the experimentally validated targets of the down-regulated miRNAs.



GO, gene ontology.

We then looked for which of the up-regulated miRNAs target genes are reported to be associated with neuroblastoma and melanoma, two tumors that affect cells originating from the neural crest, such as thyroid C-cells. We have found that *NF1, C3, MAP3K12, SYNE2, VANGL1* are targets associated with neuroblastoma, while *NF1, TP53, CCDC28A, DDX3X, DSC1, LRP1B, MCMBP, NMRK2, OXA1L, PCDHB8, PLCB4, PTPRO, SLC17A5, SLCO1B1 e UBB* are targets associated with melanoma.

Repeating the same analysis with the down-regulated miRNAs target genes, we found that *KRAS, ATRX, ARID1A, NF1, SLC12A6, PTPN11, PLXND1, HERC1, HERC2 e SYNE2* are associated with neuroblastoma, while *ACD, PTEN, CDKN2A, NF1, CTNNB1, RAC1, CCDC28A, GRIN2A, TP53, PPIAL4G, G3BP1, KNSTRN, GNAI2, OXA1L, IDH1, RB1, DDX3X, RPS27, PPP6C, UBB, TNFSF10, PTPRK, WDR12, SMARCB1, UBE2D1, TACC1, VXN* e *CNOT9* are associated with melanoma.

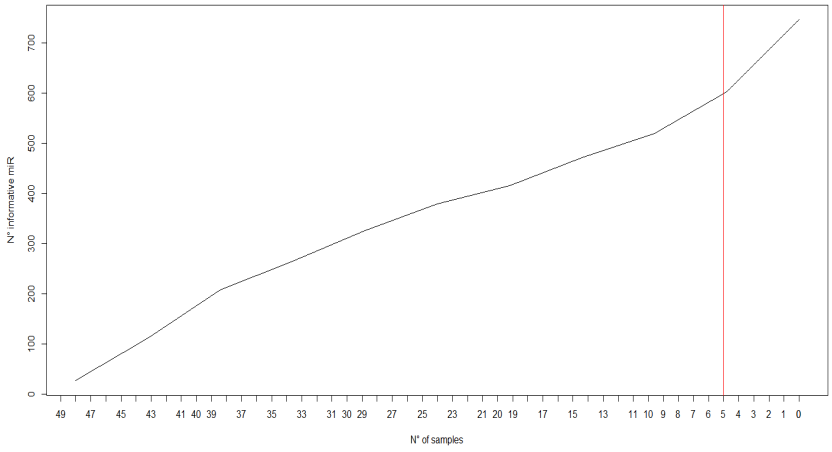
We then investigated which up-regulated and down-regulated miRNAs target genes are also associated with other neuroendocrine tumors. Among the genes target of up-regulated miRNAs we found *CDKN1A, CD44, ABCG2, TP53, BMP2, CAV1, FN1, PDGFRA, TYMS, ATM, HMGA2, ESR1, AKT1, VEGFA, ATAT1, C1QTNF1, CCND1, CCNE1, CDK6, GGH, GHSR, HSPA4, IAPP, IL12A, LGALSL, NFKB1, NKX2-2, PCNA, PIK3CG, PKD2, POU4F1 e SDHB*.

Among the target genes of the down-regulated miRNAs we found: *PNMA2, NTRK3, FHIT, BCL2, CDKN1B, CDKN1A, CDKN2A, AMACR, CD44, CD34, ETS1, ERBB2, SGFR, GLI1, TP53, KIT, DEK, IGF2BP3, NEUROD1, VCL, PLAU, TERT, TYMS, CCND1, EWSR1, PAX6, HES1, KITLG, CKAP4, NES, UBE2C, KRT19, UVRAG, BAX, KRAS, PTEN, BCL2L11, AKT1, ATM, APC, ATRX, CDK4, CCNE1, DAXX, ARID1A, CALR, ESR1, NOTCH1, PIK3CD, IGF1R, FZD4, HMGA1, HMGA2, VEGFA, MTOR, IGF2, CDK6, ODC1, GNAI2, GHSR, GNAS, MUC1, RB1, KIF5B, HSPA4, MAPK1, RAF1, MET, PCNA, ELK3, SDHB, VHL, TSC1, RET, SLC1A5, SLC7A5, TRPV1 e TM7SF2*.

4. *Step 2: identification of miRNAs expressed in both plasma and tumor tissues of MTC patients (Group 1a vs Group 1b)*

Starting from the analysis of the 754 miRNAs and considering as described above the miRNAs expressed in at least 10% of the samples, we found that 603 miRNAs are considered to be informative, comparing pre-operative plasma from each MTC patients (Group 1a) to tumor tissue of the same patient (Group 1b) (**Figure 6**).

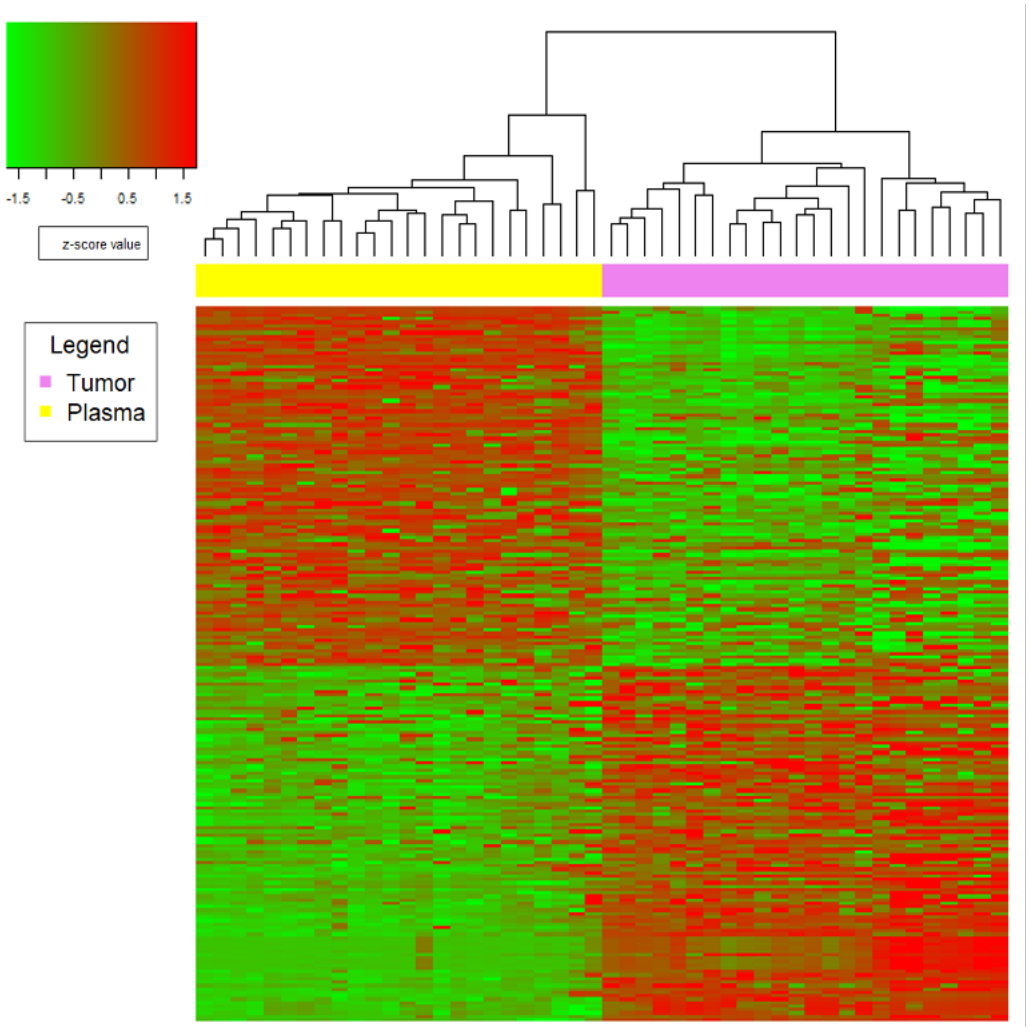
Figure 6. Informative miRNAs in MTC patients (Group 1a *vs* Group 1b).



	nInformativeMiR	perc
0%	746	0.0
10%	603	4.8
20%	519	9.6
30%	471	14.4
40%	415	19.2
50%	379	24.0
60%	326	28.8
70%	265	33.6
80%	208	38.4
90%	112	43.2
100%	27	48.0

Among these 603 miRNAs, 209 were differentially expressed between Group 1a and Group 1b. Hierarchical clustering analysis of these differentially expressed miRNAs and plasma and tumor samples are presented in **Figure 7**. In particular, 104 miRNAs were up-regulated and 105 miRNAs were down-regulated in Group 1a compared to Group 1b. The remaining 394 miRNAs were equally expressed in plasma and tumor samples of the same MTC patient.

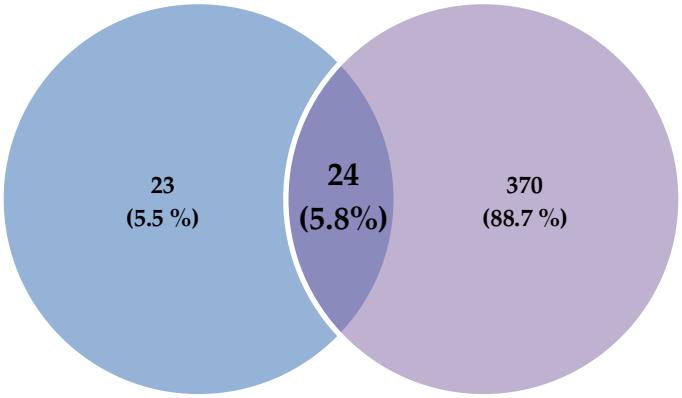
Figure 7. Heatmap of the 209 differentially expressed miRNAs in Group 1a *vs* Group 1b. The upper color bar represents sample classes; yellow represents MTC patients blood samples and pink represents MTC patients tumor samples.



5. *Step 3: combining data from Step 1 and Step 2 analysis*

Combining the results obtained from Step 1 (differentially expressed pEV-miRNAs in MTC patients - Group 1a – *vs* healthy subjects - Group 2 -) and the results obtained from Step 2 (tumor-derived pEV-miRNAs), we identified 24 miRNAs (**Figure 8**). These 24 miRNAs represent potentially diagnostic biomarkers in MTC.

Figure 8. Venn diagram of overlapping miRNAs between miRNAs differentially expressed in MTC patients and healthy controls and miRNAs equally expressed in MTC patients' tumor tissues and plasma.



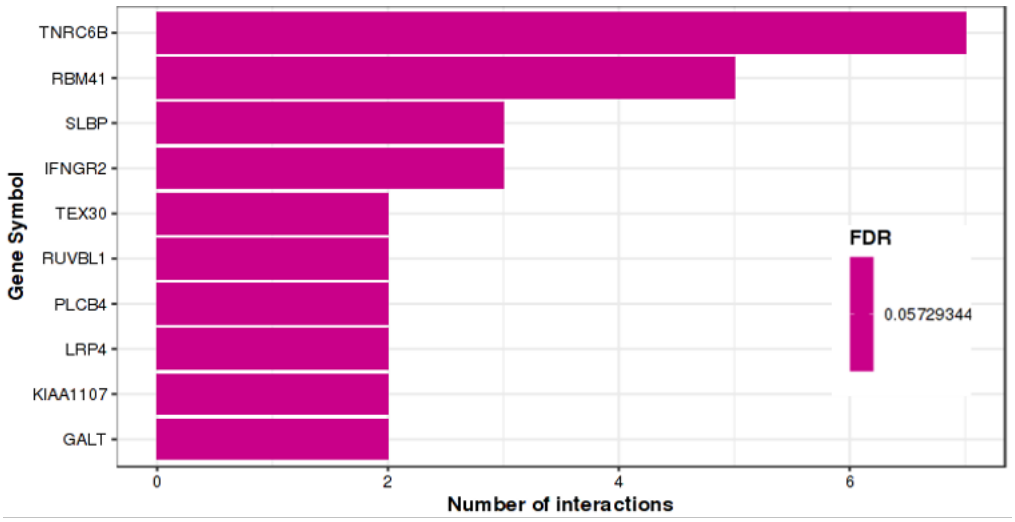
In particular, among these 24 identified miRNAs, 14 of them are up-regulated and 10 of them are down-regulated (**Table 5**).

Table 5. Up-regulated and down-regulated miRNAs deriving from Step 1 and Step 2 analysis (differentially expressed miRNAs in MTC patients and healthy controls and equally expressed miRNAs in MTC patients' samples).

Up-regulated miRNAs		**Down-regulated miRNAs**
hsa_miR_7_2_3p_478199_mir		hsa_let_7d_3p_477848_mir
hsa_miR_26b_5p_478418_mir		hsa_miR_10b_5p_478494_mir
hsa_miR_101_5p_478620_mir		hsa_miR_30a_5p_479448_mir
hsa_miR_187_3p_477941_mir		hsa_miR_183_5p_477937_mir
hsa_miR_197_3p_477959_mir		hsa_miR_217_478773_mir
hsa_miR_329_3p_478029_mir		hsa_miR_296_5p_477836_mir
hsa_miR_370_3p_478326_mir		hsa_miR_320a_478594_mir
hsa_miR_373_3p_478363_mir		hsa_miR_423_5p_478090_mir
hsa_miR_412_3p_478087_mir		hsa_miR_450b_5p_478914_mir
hsa_miR_450b_3p_478913_mir		hsa_miR_532_5p_478151_mir
hsa_miR_454_3p_478329_mir		
hsa_miR_520d_5p_478616_mir		
hsa_miR_548j_5p_479022_mir		
hsa_miR_654_3p_479135_mir		

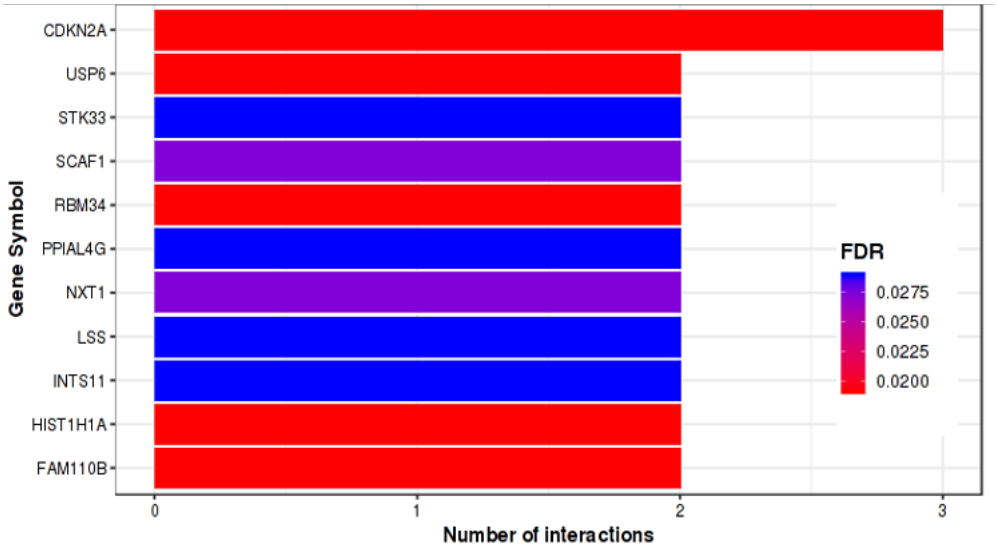
The enrichment analysis of the target genes of the up-regulated miRNAs shows that *TNRC6B* is the most targeted gene. In particular, 7/14 (50%) of the up-regulated miRNAs target *TNRC6B*, although the FDR is at the limit of statistical significance (**Figure 9**).

Figure 9. Enrichment analysis of the up-regulated miRNAs targeted genes.



We performed the same enrichment analysis for the targeted genes of the down-regulated miRNAs. A total of 11 targeted genes of at least 2 miRNAs were identified, with a statistically significant FDR (**Figure 10**). In particular, we identified *CDKN2A* as the targeted gene of 3/10 (30%) miRNAs.

Figure 10. Enrichment analysis of the down-regulated miRNAs targeted genes.



Finally, the enrichment analysis for the most significant targets of all 24 miRNAs identified various biological processes (**Figure 11**). Among these, the most significant are represented by the cellular response to stress, the response to hydrogen peroxide, the regulation of the cell cycle, and metabolic processes.

Figure 11. Functional enrichment analysis of de-regulated miRNAs targeted genes.

Biological Process (GO)			
GO-term	*description*	*count in gene set*	*false discovery r*
GO:0033554	cellular response to stress	35 of 1553	0.00059
GO:0042542	response to hydrogen peroxide	9 of 112	0.0017
GO:0051726	regulation of cell cycle	27 of 1129	0.0022
GO:0044238	primary metabolic process	106 of 8808	0.0025
GO:0044237	cellular metabolic process	106 of 8797	0.0025
GO:0071704	organic substance metabolic process	108 of 9135	0.0032
GO:0070647	protein modification by small protein conjugation or removal	23 of 945	0.0043
GO:0009893	positive regulation of metabolic process	51 of 3280	0.0043
GO:0008152	metabolic process	111 of 9569	0.0043
GO:0006950	response to stress	51 of 3267	0.0043
GO:0000302	response to reactive oxygen species	10 of 189	0.0043
GO:0051173	positive regulation of nitrogen compound metabolic process	47 of 2946	0.0049
GO:0031325	positive regulation of cellular metabolic process	48 of 3060	0.0055
GO:0010604	positive regulation of macromolecule metabolic process	48 of 3081	0.0061
GO:0071310	cellular response to organic substance	38 of 2219	0.0068
GO:0006979	response to oxidative stress	13 of 373	0.0068
GO:0006139	nucleobase-containing compound metabolic process	63 of 4551	0.0068
GO:0044271	cellular nitrogen compound biosynthetic process	52 of 3528	0.0087
GO:0009142	nucleoside triphosphate biosynthetic process	7 of 103	0.0087
GO:0010033	response to organic substance	44 of 2815	0.0098
GO:0048518	positive regulation of biological process	71 of 5459	0.0102
GO:1901576	organic substance biosynthetic process	63 of 4656	0.0105
GO:1901360	organic cyclic compound metabolic process	66 of 4963	0.0105
GO:0070887	cellular response to chemical stimulus	42 of 2672	0.0105
GO:0044249	cellular biosynthetic process	62 of 4567	0.0105
GO:0002431	Fc receptor mediated stimulatory signaling pathway	6 of 75	0.0105
GO:0009987	cellular process	147 of 14652	0.0113
GO:1990314	cellular response to insulin-like growth factor stimulus	3 of 9	0.0121
GO:0071840	cellular component organization or biogenesis	69 of 5342	0.0121
GO:0071396	cellular response to lipid	14 of 486	0.0121
GO:0051641	cellular localization	36 of 2180	0.0121
GO:0045935	positive regulation of nucleobase-containing compound m...	31 of 1770	0.0121

FDR <0.05

Discussion

Patients with MTC have worse outcomes with respect to the most frequent differentiated thyroid cancers that originate in the follicular cells such as papillary and follicular carcinomas.

There are clinical aspects still not settled for MTC. Approximately 50% of patients present with stage III/IV disease for lack of early diagnostic tools; patients with sporadic disease often present lymph node metastases at the diagnosis, with a decreased survival rate; to date, despite new and promising emerging therapies, there are no sensitive biomarkers of response to the current available treatments.

Advances in genetic testing have revolutionized the management of MTC. Indeed, in all patients genetic screening of the *RET* mutations today is a milestone for a correct clinical approach to investigate disease inheritance or to identify specific somatic mutations that correlate with worse prognosis (Romei, *et al.* 2016b).

Over the last few years, several studies have reported increasing attention intending to identify tumor heterogeneity. Liquid biopsy represents an emerging and non-invasive promising tool in diagnosis, prognosis, and personalized medicine, with the aim to identify new circulating biomarkers in MTC patients with particular reference to early diagnosis, prediction of surgery outcome, and response to treatment (Khatami, *et al.* 2019).

In this scenario, apart from genetic drive alteration, miRNAs have been recently identified as new key players in tumorigenesis and may provide a valuable resource. Therefore, the determination of miRNAs in tumors could be an important tool for a better molecular characterization of MTC patients and their possible use as biomarkers (Chen, *et al.* 2008).

Indeed, recent studies have reported miRNAs deregulation in MTC samples (Chu and Lloyd 2016). Unfortunately, limited studies are presented on tissue miRNAs expression in MTC and their clinical significance (Duan, *et al.* 2014; Gundara, *et al.* 2014; Mian, *et al.* 2012; Pennelli, *et al.* 2015; Romeo, *et al.* 2018; Spitschak, *et al.* 2017).

A recently published systematic review and meta-analysis by Silaghi et al. summarize the current knowledge regarding the prognostic impact of tissue and plasma miRNAs in

thyroid cancers, including MTC (Silaghi, *et al.* 2020). In particular, the overexpression of miRs-21, 183, and 375 in MTC are predictors of persistence, lateral lymph node metastases, residual disease, distant metastases, and mortality (Abraham, *et al.* 2011; Galuppini, *et al.* 2017; Pennelli, *et al.* 2015). The subexpression of miR-224 is a predictor of persistence and progression (Cavedon, *et al.* 2017; Mian, *et al.* 2012).

EVs are a consistent source of tumor-derived RNA due to their prevalence in circulating bodily fluids. Recent results in other types of cancer suggest that information derived from analysis of EVs from peripheral blood plasma can be integrated into the routine diagnostic tumor approach (Rappa, *et al.* 2019). MiRNAs present in extracellular plasma vesicles (pEV-miRNAs) are enriched and better protected from degradation. Furthermore, they are actively released, and they are thought to act as hormones with a paracrine and endocrine effect. Unfortunately, no studies are available focusing on pEV-miRNAs in MTC.

In this multicenter observational study, we prospectively enrolled patients with MTC at the time of diagnosis, intending to find new diagnostic biomarkers as a tool to be used in the several phases of the disease.

The study was translationally conducted through 4 steps. First of all, a group of healthy subjects was matched to MTC patients at the time of the diagnosis and pre-operative plasma of MTC patients was compared to the plasma of age and sex-matched healthy controls: 44 pEV-miRNAs (21 up-regulated and 23 down-regulated) were identified as differentially expressed between the two conditions (disease *vs* health). In addition, pre-operative plasma and tumor tissue of the same MTC patient were compared and 394 pEV-miRNAs were identified as tumor-derived. In the final step, data of the previous phases were analyzed and compared, and allowed to finally identify 24 miRNAs (14 up-regulated and 10 down-regulated) as potential diagnostic biomarkers in MTC. Among the up-regulated miRNAs, hsa_miR_7_2_3p, hsa_miR_370_3p, and hsa_miR_373_3p have been reported in the literature as tissue miRNAs associated with MTC (Chu and Lloyd 2016; Fu, *et al.* 2017). Among the down-regulated miRNAs, hsa_miR_296_6p has been reported in the literature associated with MTC (Fu, *et al.* 2017). The remaining up- and down-regulated miRNAs are

not described in the literature associated with MTC and this study represents the basis for further investigating the role of these miRNAs in this type of thyroid cancer.

The functional enrichment analysis allowed to identify that targeted genes of the deregulated miRNAs identified in this study are common with other neuroendocrine tumors, such as neuroblastoma and melanoma.

Additionally, the functional enrichment analysis of the 24 up- and down-regulated miRNAs allowed us to identify specifically targeted genes and signaling pathways involved in key roles biological processes, such as cellular response to stress, the response to hydrogen peroxide, the regulation of the cell cycle, and metabolic processes. In particular, *TNRC6B*, a gene that regulates gene expression by stabilizing miRNAs itself, is the most targeted gene of the up-regulated miRNAs. There are no data in the literature regarding TNRC6B and MTC. Among the other targeted genes of the up-regulated miRNAs, there are: *RBM41*, a protein-coding gene that controls RNA splicing; *SLBP*, a protein-coding gene that is known to control histone metabolism by regulating chromatin structure; *RUVBL1*, a protein-coding gene that controls transcription by activating it. Among the targeted genes of the down-regulated miRNAs, *CDKN2A*, a CDK4 inhibitor that induces cell cycle arrest, is the most frequently targeted gene. Alterations of genes involved in the G1 phase of the cell cycle, including the cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors, are common events in the neoplastic development of a series of different types of tumors. Their role in sporadic MTC is still largely little known (Barbieri*, et al.* 2014). Among the other targeted genes of the down-regulated miRNAs, there are: *SCAF1*, a protein-coding gene that may control RNA splicing; *PPIAL4G*, a protein-coding gene that accelerates the folding of proteins; *NXT1*, a protein-coding gene that controls the nuclear export of mRNA; *INTS11*, a protein-coding gene that controls the small nuclear RNAs involved in the splicing process; *HIST1H1A*, a gene that encodes a member of the histone H1 family by regulating chromatin structure. Therefore, these 24 up- and down-regulated miRNAs could play an active role in the modulation of significant cellular pathways in targeted cells.

This study has some limitations. The number of patients with MTC is limited, but we are in the context of a rare disease. Given the rarity of this malignancy, collaboration with multiple referral centers for MTC is essential. Furthermore, to date, no studies are present on pEV-miRNAs in MTC and this is the first study focused on this topic.

Conclusions

The present study allowed to identify a specific pattern of pEV-miRNAs in patients with MTC at the time of diagnosis. This pattern partially reflects the tissue miRNA expression profile of tumor samples.

Liquid biopsy by searching these MTC-specific miRNAs may represent a new and promising frontier for the discovery of unknown mechanisms involved in MTC and the detection of prognostic and predictive biomarkers in this disease. The definition of the molecular and mutational profile for each MTC patient may predict the outcome and identify the drug response /resistance profile.

The 24 MTC-specific miRNAs identified in this study represent potential diagnostic biomarkers in patients with MTC. In particular, this study aims to be a pilot observational study to identify new biomarkers in MTC, with particular reference to early diagnosis.

Once established that these circulating miRNAs may represent specific disease biomarkers, their evaluation could be used to follow-up the course of the disease, with a particular focus on cure and progression. In case of disease progression, the use of these circulating innovative biomarkers and the difference in their expression could be useful to improve prognostic accuracy and better define the response or resistance to treatment and the clinicians' decisions in MTC patients' management.

Thus, this study may represent the springboard for future studies for validating these preliminary data and identifying new biomarkers that can be used during the follow-up of patients with MTC treated with tailored therapies.

References

Abraham D, Jackson N, Gundara JS, Zhao J, Gill AJ, Delbridge L, Robinson BG & Sidhu SB 2011 MicroRNA profiling of sporadic and hereditary medullary thyroid cancer identifies predictors of nodal metastasis, prognosis, and potential therapeutic targets. *Clin Cancer Res* 17 4772-4781.

Accardo G, Conzo G, Esposito D, Gambardella C, Mazzella M, Castaldo F, Di Donna C, Polistena A, Avenia N, Colantuoni V, et al. 2017 Genetics of medullary thyroid cancer: An overview. *Int J Surg* 41 Suppl 1 S2-s6.

Al-Jundi M, Thakur S, Gubbi S & Klubo-Gwiezdzinska J 2020 Novel Targeted Therapies for Metastatic Thyroid Cancer-A Comprehensive Review. *Cancers (Basel)* 12.

Amin MB, Greene FL, Edge SB, Compton CC, Gershenwald JE, Brookland RK, Meyer L, Gress DM, Byrd DR & Winchester DP 2017 The Eighth Edition AJCC Cancer Staging Manual: Continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging. *CA Cancer J Clin* 67 93-99.

Aubert S, Berdelou A, Gnemmi V, Behal H, Caiazzo R, D'Herbomez M, Pigny P, Wemeau JL, Carnaille B, Renaud F, et al. 2018 Large sporadic thyroid medullary carcinomas: predictive factors for lymph node involvement. *Virchows Arch* 472 461-468.

Barbet J, Campion L, Kraeber-Bodere F & Chatal JF 2005 Prognostic impact of serum calcitonin and carcinoembryonic antigen doubling-times in patients with medullary thyroid carcinoma. *J Clin Endocrinol Metab* 90 6077-6084.

Barbieri RB, Bufalo NE, Secolin R, Assumpção LV, Maciel RM, Cerutti JM & Ward LS 2014 Polymorphisms of cell cycle control genes influence the development of sporadic medullary thyroid carcinoma. *Eur J Endocrinol* 171 761-767.

Bastholt L, Kreissl MC, Fuhrer D, Maia AL, Locati LD, Maciel L, Wu Y, Heller KN, Webster A & Elisei R 2016 Effect of an Outreach Programme on Vandetanib Safety in Medullary Thyroid Cancer. *Eur Thyroid J* 5 187-194.

Bodei L, Handkiewicz-Junak D, Grana C, Mazzetta C, Rocca P, Bartolomei M, Lopera Sierra M, Cremonesi M, Chinol M, Macke HR, et al. 2004 Receptor radionuclide therapy with 90Y-DOTATOC in patients with medullary thyroid carcinomas. *Cancer Biother Radiopharm* 19 65-71.

Boichard A, Croux L, Al Ghuzlan A, Broutin S, Dupuy C, Leboulleux S, Schlumberger M, Bidart JM & Lacroix L 2012 Somatic RAS mutations occur in a large proportion of sporadic RET-negative medullary thyroid carcinomas and extend to a previously unidentified exon. *J Clin Endocrinol Metab* 97 E2031-2035.

Brierley J, Gospodarowicz MK & Wittekind C 2017 *TNM classification of malignant tumours*. Chichester, West Sussex, UK ; Hoboken, NJ: John Wiley & Sons, Inc.

Burrell RA & Swanton C 2014 Tumour heterogeneity and the evolution of polyclonal drug resistance. *Mol Oncol* 8 1095-1111.

Cabanillas ME, Hu MI & Jimenez C 2014 Medullary thyroid cancer in the era of tyrosine kinase inhibitors: to treat or not to treat--and with which drug--those are the questions. *J Clin Endocrinol Metab* 99 4390-4396.

Cavedon E, Barollo S, Bertazza L, Pennelli G, Galuppini F, Watutantrige-Fernando S, Censi S, Iacobone M, Benna C, Vianello F, et al. 2017 Prognostic Impact of miR-224 and RAS Mutations in Medullary Thyroid Carcinoma. *Int J Endocrinol* 2017 4915736.

Celano M, Mignogna C, Rosignolo F, Sponziello M, Iannone M, Lepore SM, Lombardo GE, Maggisano V, Verrienti A, Bulotta S, et al. 2018 Expression of YAP1 in aggressive thyroid cancer. In *Endocrine*, pp 209-212. United States.

Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, Zhang Y, Chen J, Guo X, et al. 2008 Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res* 18 997-1006.

Chernock RD & Hagemann IS 2015 Molecular pathology of hereditary and sporadic medullary thyroid carcinomas. *Am J Clin Pathol* 143 768-777.

Cho U, Oh WJ, Bae JS, Lee S, Lee YS, Park GS & Jung CK 2014 Clinicopathological features of rare BRAF mutations in Korean thyroid cancer patients. *J Korean Med Sci* 29 1054-1060.

Chu YH & Lloyd RV 2016 Medullary Thyroid Carcinoma: Recent Advances Including MicroRNA Expression. *Endocr Pathol* 27 312-324.

Ciampi R, Romei C, Cosci B, Vivaldi A, Bottici V, Renzini G, Ugolini C, Tacito A, Basolo F, Pinchera A, et al. 2012 Chromosome 10 and RET gene copy number alterations in hereditary and sporadic Medullary Thyroid Carcinoma. *Mol Cell Endocrinol* 348 176-182.

Ciampi R, Romei C, Ramone T, Prete A, Tacito A, Cappagli V, Bottici V, Viola D, Torregrossa L, Ugolini C, et al. 2019 Genetic Landscape of Somatic Mutations in a Large Cohort of Sporadic Medullary Thyroid Carcinomas Studied by Next-Generation Targeted Sequencing. *iScience* 20 324-336.

Ciampi R, Mian C, Fugazzola L, Cosci B, Romei C, Barollo S, Cirello V, Bottici V, Marconcini G, Rosa PM, et al. 2013 Evidence of a low prevalence of RAS mutations in a large medullary thyroid cancer series. *Thyroid* 23 50-57.

Collins BT, Cramer HM, Tabatowski K, Hearn S, Raminhos A & Lampe H 1995 Fine needle aspiration of medullary carcinoma of the thyroid. Cytomorphology, immunocytochemistry and electron microscopy. *Acta Cytol* 39 920-930.

Dilts DM, Sandler A, Cheng S, Crites J, Ferranti L, Wu A, Gray R, MacDonald J, Marinucci D & Comis R 2008 Development of clinical trials in a cooperative group setting: the eastern cooperative oncology group. *Clin Cancer Res* 14 3427-3433.

Duan L, Hao X, Liu Z, Zhang Y & Zhang G 2014 MiR-129-5p is down-regulated and involved in the growth, apoptosis and migration of medullary thyroid carcinoma cells through targeting RET. *FEBS Lett* 588 1644-1651.

Dvorakova S, Vaclavikova E, Ryska A, Cap J, Vlcek P, Duskova J, Kodetova D, Holub V, Novak Z & Bendlova B 2006 Double germline mutations in the RET Proto-oncogene in MEN 2A and MEN 2B kindreds. *Exp Clin Endocrinol Diabetes* 114 192-196.

Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, et al. 2009 New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 45 228-247.

Elisei R, Alevizaki M, Conte-Devolx B, Frank-Raue K, Leite V & Williams GR 2013a 2012 European thyroid association guidelines for genetic testing and its clinical consequences in medullary thyroid cancer. *Eur Thyroid J* 1 216-231.

Elisei R, Romei C, Renzini G, Bottici V, Cosci B, Molinaro E, Agate L, Cappagli V, Miccoli P, Berti P, et al. 2012 The timing of total thyroidectomy in RET gene mutation carriers could be personalized and safely planned on the basis of serum calcitonin: 18 years experience at one single center. *J Clin Endocrinol Metab* 97 426-435.

Elisei R, Schlumberger MJ, Muller SP, Schoffski P, Brose MS, Shah MH, Licitra L, Jarzab B, Medvedev V, Kreissl MC, et al. 2013b Cabozantinib in progressive medullary thyroid cancer. *J Clin Oncol* 31 3639-3646.

Fox E, Widemann BC, Chuk MK, Marcus L, Aikin A, Whitcomb PO, Merino MJ, Lodish M, Dombi E, Steinberg SM, et al. 2013 Vandetanib in children and adolescents with multiple endocrine neoplasia type 2B associated medullary thyroid carcinoma. *Clin Cancer Res* 19 4239-4248.

Frank-Raue K & Raue F 2015 Hereditary Medullary Thyroid Cancer Genotype-Phenotype Correlation. *Recent Results Cancer Res* 204 139-156.

Fu X, Fang J, Lian M, Zhong Q, Ma H, Feng L, Wang R & Wang H 2017 Identification of microRNAs associated with medullary thyroid carcinoma by bioinformatics analyses. *Mol Med Rep* 15 4266-4272.

Galuppini F, Bertazza L, Barollo S, Cavedon E, Rugge M, Guzzardo V, Sacchi D, Watutantrige-Fernando S, Vianello F, Mian C, et al. 2017 MiR-375 and YAP1 expression profiling in medullary thyroid carcinoma and their correlation with clinical-pathological features and outcome. *Virchows Arch* 471 651-658.

Goutas N, Vlachodimitropoulos D, Bouka M, Lazaris AC, Nasioulas G & Gazouli M 2008 BRAF and K-RAS mutation in a Greek papillary and medullary thyroid carcinoma cohort. *Anticancer Res* 28 305-308.

Gundara JS, Zhao JT, Gill AJ, Clifton-Bligh R, Robinson BG, Delbridge L & Sidhu SB 2014 Nodal metastasis microRNA expression correlates with the primary tumour in MTC. *ANZ J Surg* 84 235-239.

Hadoux J & Schlumberger M 2017 Chemotherapy and tyrosine-kinase inhibitors for medullary thyroid cancer. *Best Pract Res Clin Endocrinol Metab* 31 335-347.

Hadoux J, Pacini F, Tuttle RM & Schlumberger M 2016 Management of advanced medullary thyroid cancer. *Lancet Diabetes Endocrinol* 4 64-71.

Hazard JB, Hawk WA & Crile G, Jr. 1959 Medullary (solid) carcinoma of the thyroid; a clinicopathologic entity. *J Clin Endocrinol Metab* 19 152-161.

Heilmann AM, Subbiah V, Wang K, Sun JX, Elvin JA, Chmielecki J, Sherman SI, Murthy R, Busaidy NL, Subbiah I, et al. 2016 Comprehensive Genomic Profiling of Clinically Advanced Medullary Thyroid Carcinoma. *Oncology* 90 339-346.

Hudson J, Duncavage E, Tamburrino A, Salerno P, Xi L, Raffeld M, Moley J & Chernock RD 2013 Overexpression of miR-10a and miR-375 and downregulation of YAP1 in medullary thyroid carcinoma. *Exp Mol Pathol* 95 62-67.

Iten F, Muller B, Schindler C, Rochlitz C, Oertli D, Macke HR, Muller-Brand J & Walter MA 2007 Response to [90Yttrium-DOTA]-TOC treatment is associated with long-term survival benefit in metastasized medullary thyroid cancer: a phase II clinical trial. *Clin Cancer Res* 13 6696-6702.

Jasim S, Ying AK, Waguespack SG, Rich TA, Grubbs EG, Jimenez C, Hu MI, Cote G & Habra MA 2011 Multiple endocrine neoplasia type 2B with a RET proto-oncogene A883F mutation displays a more indolent form of medullary thyroid carcinoma compared with a RET M918T mutation. *Thyroid* 21 189-192.

Ji JH, Oh YL, Hong M, Yun JW, Lee HW, Kim D, Ji Y, Kim DH, Park WY, Shin HT, et al. 2015 Identification of Driving ALK Fusion Genes and Genomic Landscape of Medullary Thyroid Cancer. *PLoS Genet* 11 e1005467.

Jiang M, Shi X, Zhu H, Wei W & Li J 2019 Two GEO MicroRNA Expression Profile Based High-Throughput Screen to Identify MicroRNA-31-3p Regulating Growth of Medullary Thyroid Carcinoma Cell by Targeting RASA2. *Med Sci Monit* 25 5170-5180.

Kasaian K, Wiseman SM, Walker BA, Schein JE, Hirst M, Moore RA, Mungall AJ, Marra MA & Jones SJ 2016 Putative BRAF activating fusion in a medullary thyroid cancer. *Cold Spring Harb Mol Case Stud* 2 a000729.

Khatami F, Larijani B, Nasiri S & Tavangar SM 2019 Liquid Biopsy as a Minimally Invasive Source of Thyroid Cancer Genetic and Epigenetic Alterations. *Int J Mol Cell Med* 8 19-29.

Laure Giraudet A, Al Ghulzan A, Auperin A, Leboulleux S, Chehboun A, Troalen F, Dromain C, Lumbroso J, Baudin E & Schlumberger M 2008 Progression of medullary thyroid carcinoma: assessment with calcitonin and carcinoembryonic antigen doubling times. *Eur J Endocrinol* 158 239-246.

Machens A & Dralle H 2013 Benefit-risk balance of reoperation for persistent medullary thyroid cancer. *Ann Surg* 257 751-757.

Machens A & Dralle H 2018 Advances in risk-oriented surgery for multiple endocrine neoplasia type 2. *Endocr Relat Cancer* 25 T41-t52.

Machens A, Lorenz K, Weber F & Dralle H 2018 Genotype-specific progression of hereditary medullary thyroid cancer. *Hum Mutat* 39 860-869.

Meijer JA, le Cessie S, van den Hout WB, Kievit J, Schoones JW, Romijn JA & Smit JW 2010 Calcitonin and carcinoembryonic antigen doubling times as prognostic factors in medullary thyroid carcinoma: a structured meta-analysis. *Clin Endocrinol (Oxf)* 72 534-542.

Mian C, Pennelli G, Fassan M, Balistreri M, Barollo S, Cavedon E, Galuppini F, Pizzi M, Vianello F, Pelizzo MR, et al. 2012 MicroRNA profiles in familial and sporadic medullary thyroid carcinoma: preliminary relationships with RET status and outcome. *Thyroid* 22 890-896.

Moura MM, Cavaco BM & Leite V 2015 RAS proto-oncogene in medullary thyroid carcinoma. *Endocr Relat Cancer* 22 R235-252.

Moura MM, Cavaco BM, Pinto AE & Leite V 2011 High prevalence of RAS mutations in RET-negative sporadic medullary thyroid carcinomas. *J Clin Endocrinol Metab* 96 E863-868.

Mulligan LM 2014 RET revisited: expanding the oncogenic portfolio. *Nat Rev Cancer* 14 173-186.

Nelkin B 2017 Recent advances in the biology and therapy of medullary thyroid carcinoma. *F1000Res* 6 2184.

Pennelli G, Galuppini F, Barollo S, Cavedon E, Bertazza L, Fassan M, Guzzardo V, Pelizzo MR, Rugge M & Mian C 2015 The PDCD4/miR-21 pathway in medullary thyroid carcinoma. *Hum Pathol* 46 50-57.

Prete A, Borges de Souza P, Censi S, Muzza M, Nucci N & Sponziello M 2020 Update on Fundamental Mechanisms of Thyroid Cancer. *Front Endocrinol (Lausanne)* 11 102.

Puppin C, Durante C, Sponziello M, Verrienti A, Pecce V, Lavarone E, Baldan F, Campese AF, Boichard A, Lacroix L, et al. 2014 Overexpression of genes involved in miRNA biogenesis in medullary thyroid carcinomas with RET mutation. *Endocrine* 47 528-536.

Ramundo V, Milone F, Severino R, Savastano S, Di Somma C, Vuolo L, De Luca L, Lombardi G, Colao A & Faggiano A 2011 Clinical and prognostic implications of the genetic diagnosis of hereditary NET syndromes in asymptomatic patients. *Horm Metab Res* 43 794-800.

Rappa G, Puglisi C, Santos MF, Forte S, Memeo L & Lorico A 2019 Extracellular Vesicles from Thyroid Carcinoma: The New Frontier of Liquid Biopsy. *Int J Mol Sci* 20.

Raue F & Frank-Raue K 2018 Update on Multiple Endocrine Neoplasia Type 2: Focus on Medullary Thyroid Carcinoma. *J Endocr Soc* 2 933-943.

Roman S, Lin R & Sosa JA 2006 Prognosis of medullary thyroid carcinoma: demographic, clinical, and pathologic predictors of survival in 1252 cases. *Cancer* 107 2134-2142.

Romei C, Ciampi R & Elisei R 2016a A comprehensive overview of the role of the RET proto-oncogene in thyroid carcinoma. *Nat Rev Endocrinol* 12 192-202.

Romei C, Casella F, Tacito A, Bottici V, Valerio L, Viola D, Cappagli V, Matrone A, Ciampi R, Piaggi P, et al. 2016b New insights in the molecular signature of advanced medullary thyroid cancer: evidence of a bad outcome of cases with double. *J Med Genet* 53 729-734.

Romeo P, Colombo C, Granata R, Calareso G, Gualeni AV, Dugo M, De Cecco L, Rizzetti MG, Zanframundo A, Aiello A, et al. 2018 Circulating miR-375 as a novel prognostic marker for metastatic medullary thyroid cancer patients. *Endocr Relat Cancer* 25 217-231.

Romero-Lluch AR, Cuenca-Cuenca JI, Guerrero-Vazquez R, Martinez-Ortega AJ, Tirado-Hospital JL, Borrego-Dorado I & Navarro-Gonzalez E 2017 Diagnostic utility of PET/CT with (18)F-DOPA and (18)F-FDG in persistent or recurrent medullary thyroid carcinoma: the importance of calcitonin and carcinoembryonic antigen cutoff. *Eur J Nucl Med Mol Imaging* 44 2004-2013.

Schlumberger M, Elisei R, Muller S, Schoffski P, Brose M, Shah M, Licitra L, Krajewska J, Kreissl MC, Niederle B, et al. 2017 Overall survival analysis of EXAM, a phase III trial of cabozantinib in patients with radiographically progressive medullary thyroid carcinoma. *Ann Oncol* 28 2813-2819.

Silaghi CA, Lozovanu V, Silaghi H, Georgescu RD, Pop C, Dobrean A & Georgescu CE 2020 The Prognostic Value of MicroRNAs in Thyroid Cancers-A Systematic Review and Meta-Analysis. *Cancers (Basel)* 12.

Spitschak A, Meier C, Kowtharapu B, Engelmann D & Putzer BM 2017 MiR-182 promotes cancer invasion by linking RET oncogene activated NF-kappaB to loss of the HES1/Notch1 regulatory circuit. *Mol Cancer* 16 24.

Subbiah V, Velcheti V, Tuch BB, Ebata K, Busaidy NL, Cabanillas ME, Wirth LJ, Stock S, Smith S, Lauriault V, et al. 2018a Selective RET kinase inhibition for patients with RET-altered cancers. *Ann Oncol* 29 1869-1876.

Subbiah V, Gainor JF, Rahal R, Brubaker JD, Kim JL, Maynard M, Hu W, Cao Q, Sheets MP, Wilson D, et al. 2018b Precision Targeted Therapy with BLU-667 for. *Cancer Discov* 8 836-849.

Treglia G, Rufini V, Salvatori M, Giordano A & Giovanella L 2012 PET Imaging in Recurrent Medullary Thyroid Carcinoma. *Int J Mol Imaging* 2012 324686.

Valente FO, Dias da Silva MR, Camacho CP, Kunii IS, Bastos AU, da Fonseca CC, Simiao HP, Tamanaha R, Maciel RM & Cerutti JM 2013 Comprehensive analysis of RET gene should be performed in patients with multiple endocrine neoplasia type 2 (MEN 2) syndrome and no apparent genotype-phenotype correlation: an appraisal of p.Y791F and p.C634Y RET mutations in five unrelated Brazilian families. *J Endocrinol Invest* 36 975-981.

Vuong HG, Odate T, Ngo HTT, Pham TQ, Tran TTK, Mochizuki K, Nakazawa T, Katoh R & Kondo T 2018 Clinical significance of RET and RAS mutations in sporadic medullary thyroid carcinoma: a meta-analysis. *Endocr Relat Cancer* 25 633-641.

Wells SA, Jr. 2018 Advances in the management of MEN2: from improved surgical and medical treatment to novel kinase inhibitors. *Endocr Relat Cancer* 25 T1-t13.

Wells SA, Jr., Robinson BG, Gagel RF, Dralle H, Fagin JA, Santoro M, Baudin E, Elisei R, Jarzab B, Vasselli JR, et al. 2012 Vandetanib in patients with locally advanced or metastatic medullary thyroid cancer: a randomized, double-blind phase III trial. *J Clin Oncol* 30 134-141.

Wells SA, Jr., Asa SL, Dralle H, Elisei R, Evans DB, Gagel RF, Lee N, Machens A, Moley JF, Pacini F, et al. 2015 Revised American Thyroid Association guidelines for the management of medullary thyroid carcinoma. *Thyroid* 25 567-610.

Wirth LJ, Sherman E, Robinson B, Solomon B, Kang H, Lorch J, Worden F, Brose M, Patel J, Leboulleux S, et al. 2020 Efficacy of Selpercatinib in. *N Engl J Med* 383 825-835.

Yeganeh MZ, Sheikholeslami S & Hedayati M 2015 RET proto oncogene mutation detection and medullary thyroid carcinoma prevention. *Asian Pac J Cancer Prev* 16 2107-2117.